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SPONGES DOMINANT IN THE ALASKA INTERTIDAL: BIOLOGY, ECOLOGY, AND GENETIC DIVERSITY

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

of the Degree of

DOCTOR OF PHILOSOPHY

By

Ann Lynette Knowlton, B.S.

Fairbanks, Alaska

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Abstract

The role of the sponge, *Halichondria panicea*, in a Kachemak Bay, Alaska, intertidal community was investigated through field and laboratory experiments. The relationship between *H. panicea* and co-occurring macroalgae was studied and results indicate that removing macroalgae had no effect on sponge abundance. A laboratory feeding trial investigating *H. panicea* and its primary predator *Archidoris montereyensis* showed that nudibranchs consuming symbiotic sponge had higher feeding and egg production rates than individuals eating aposymbiotic sponge. In a simulated predation event, initial sponge growth rates into experimental feeding scars were high, indicating a response mechanism to tissue damage. A naturally occurring high nudibranch recruitment into a sponge population resulted in the local decline and extinction of both sponge and predator.

Genetic studies revealed that at least two sponge species likely comprise the intertidal populations investigated, *Halichondria panicea* and *H. bowerbanki*. The reproductive cycle of *H. panicea* at exposed, hard-substrate habitats, and *H. bowerbanki* at sheltered, soft-sediment sites, exhibited seasonal peaks in oocyte production and maturation. *H. panicea* produced embryos 3-4 months earlier than *H. bowerbanki*.

Six genomic DNA microsatellite loci were isolated and utilized in the characterization of two *Halichondria panicea* populations. The two populations

were differentiated from one another with no significant inbreeding or bottleneck effect detected. All individuals were genetically unique, indicating little or no cloning. Sexual reproduction appears to be the dominant mode of reproduction maintaining the populations.

DNA sequence analyses suggest that at least two species are likely present in Kachemak Bay. Distributions of ITS and CO1 haplotypes corresponded to habitat type. Analyses of the data grouped Alaska haplotypes separately from European samples of *Halichondria panicea* and *H. bowerbanki*, suggesting separate species may occur in Alaska. A re-examination of sponge systematics in southcentral Alaska is needed.

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Chapter 1: Introduction

Modern research on intertidal communities and the mechanisms and interactions that shape them has its roots in classic experiments conducted in the 1960's and 1970's (e.g. Connell 1961, 1970; Paine 1966, 1974; Dayton 1971). Mechanisms explaining species composition, abundance, and distribution in temperate rocky intertidal communities were derived from careful observation and manipulation. A key mechanism elucidated was that space was a limiting factor and that competition (inter- and intra-specific) for available space was a major mechanism in determining community structure (Connell 1961, Dayton 1971). In addition to 'space wars', the role of predation was another major influence on the shape and structure of intertidal communities (Connell 1970, Paine 1974). Both biological and physical forces were important parts of the paradigms. A species' growth rate, ability to overgrow or shade-out others, and physiological tolerances to air exposure, sunlight, wave action, or drastic salinity changes were key in determining where it could live on the shoreline and its ability to avoid major competitors or predators. Physical factors like freezing, scouring, and desiccation could open up previously occupied space or limit the vertical mobility of predators.

While these paradigms have been useful for explaining many of the structuring mechanisms of communities, they were developed from a system that was fairly predictable. Recruitment of new individuals into a community was

consistently high and the starting conditions each year were the same. The ability of larvae to locate and settle in suitable habitats was not a factor structuring the communities. Subsequent studies in regions where recruitment is variable have revealed the importance of larval ecology (Roughgarden et al., 1986; Underwood and Fairweather 1989) and life history traits. In addition, studies in temperate regions primarily address species living in the middle of their geographic ranges where conditions are apt to be more favorable for their ecological requirements. Investigations of the same species at the extremes of their geographical distributions resulted in different responses to competition and predation pressures leading to a different community structure (Lewis et al., 1982; Wethey 1983).

Life history traits of a species, or closely related species, can vary dramatically between temperate and high latitude populations (Dehnel 1955, Clarke 1982, Highsmith and Coyle 1991). Biological interactions at high latitudes are strongly coupled with seasonal extremes in physical factors like day length, air temperature, water temperature, and ice scouring.

The coast of southcentral Alaska is a productive high latitude region located along the northern edge of the Gulf of Alaska. Cook Inlet is a prominent feature of the region that is known for its extreme tidal range and extensive mudflats. Kachemak Bay is a large embayment branching off Lower Cook Inlet and consisting of glacial fjords on its southern shore and expanses of soft-sediment beaches along its northern shore. Nutrient-rich waters from the Gulf of

Alaska enter Lower Cook Inlet along its eastern edge and flow directly into Kachemak Bay (Fig. 1.1; Trasky et al., 1977). Due to abundant nutrient availability, high summer productivity (Sambrotto and Lorenzen 1986), and continual mixing of the water column, Kachemak Bay supports highly diverse and abundant communities of marine organisms.

A unique feature of some intertidal communities in Kachemak Bay is the abundance of marine sponges. At several locations sponges are the spatial dominants, occupying more of the available substrate than any other single taxon. While several studies have shown marine sponges to be important components of some benthic communities, sponges infrequently play a dominant and conspicuous role.

Sponges are poorly studied and often misunderstood organisms. Being of a primitive animal design, sponge colonies have been described as 'loose associations of cells'. While their body structure is at the tissue level of organization with no organs or discrete body regions, sponge cells retain a high degree of plasticity in that they can change their form and function to suit the needs of the colony. General composition of a sponge is a collagen matrix (spongin fibers) embedded with rigid skeletal elements called spicules. The living cells are arrayed in and around the structural elements. Two basic growth forms are evident: thin, encrusting forms and upright branching forms. Water motion plays an influential role in determining growth morphology (Palumbi 1984, Barthel 1991).

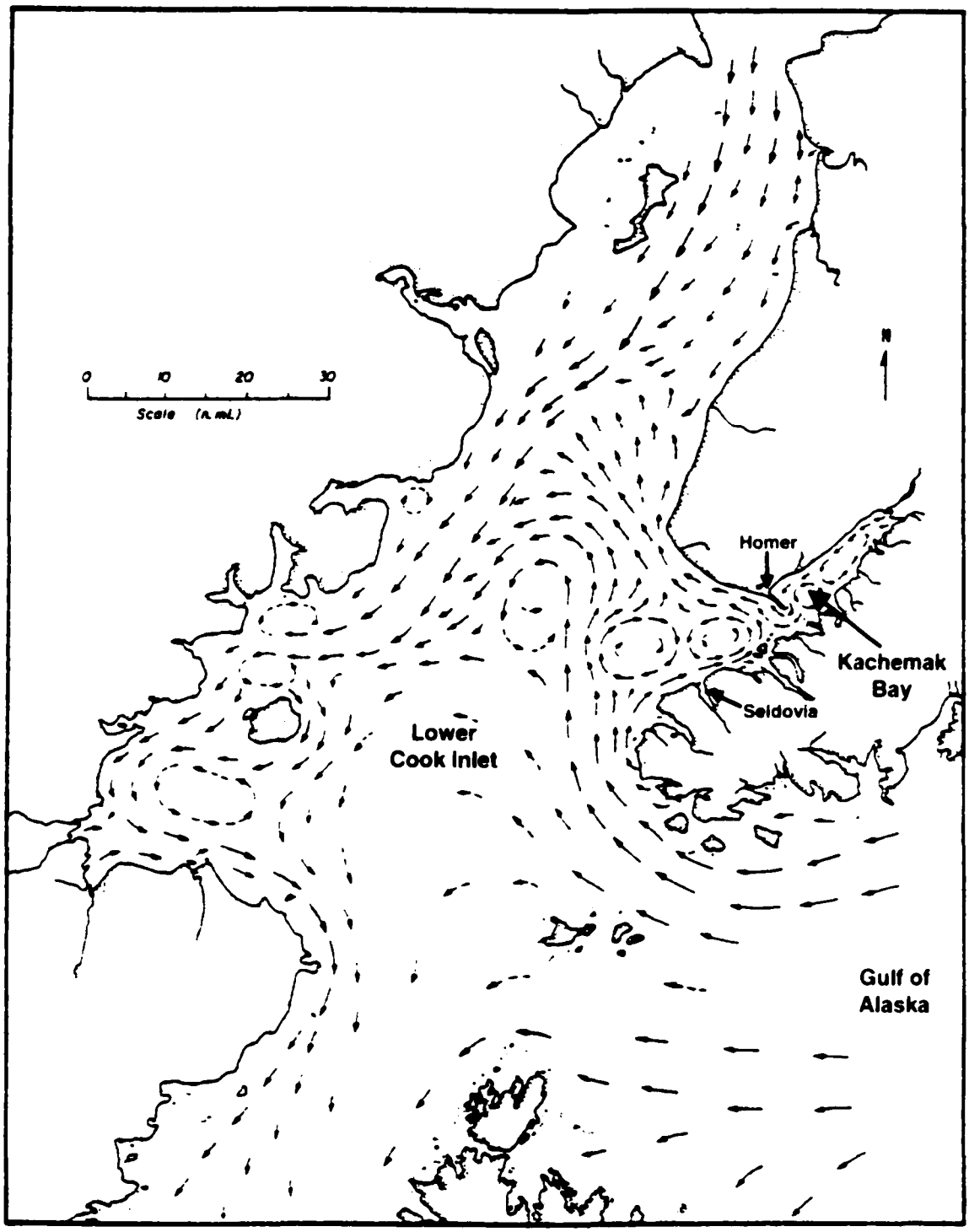

Figure 1.1: Map indicating general water circulation patterns for Lower Cook Inlet and Kachemak Bay. Diagram modified from Trasky et al. (1977).

A prominent feature of sponges is the extensive internal aquifer system. Channels through which water flows start as very small openings, called ostia, at the surface of the sponge and network together, increasing in size, until they exit the sponge through common pores called oscula (Fig. 1.2). Specialized flagellated cells called choanocytes induce water flow through the tissue. These cells are found in enlarged chambers along the aquifer system and the beating of their flagella cause water to be moved through the sponge tissue.

Water flow within sponge tissue is significant to all aspects of the organism's life. Cells are bathed in oxygenated water and able to release cellular waste products that are quickly removed. While the choanocytes are key in creating the water flow, they have other equally important functions within the colony. They capture food particles consisting primarily of bacteria and small unicellular algae and transfer the food to other cells for distribution within the sponge. In addition, choanocytes capture male gametes from the incurrent stream for sexual reproduction.

Sponges characteristically maintain symbiotic relationships with other organisms. Many sponge species host bacteria or unicellular algae within their tissues (Christensen 1985; Hinde et al., 1994; Althoff et al., 1998). The sponge acquires energy produced by the symbionts while providing a suitable place for the bacteria or algae to reside (Douglas 1988). Small invertebrates, as well as some small fish, utilize sponges as refuges for adult forms (Duffy 1992; Ilan et al., 1994) or a protected place to lay eggs (Munehara 1991).

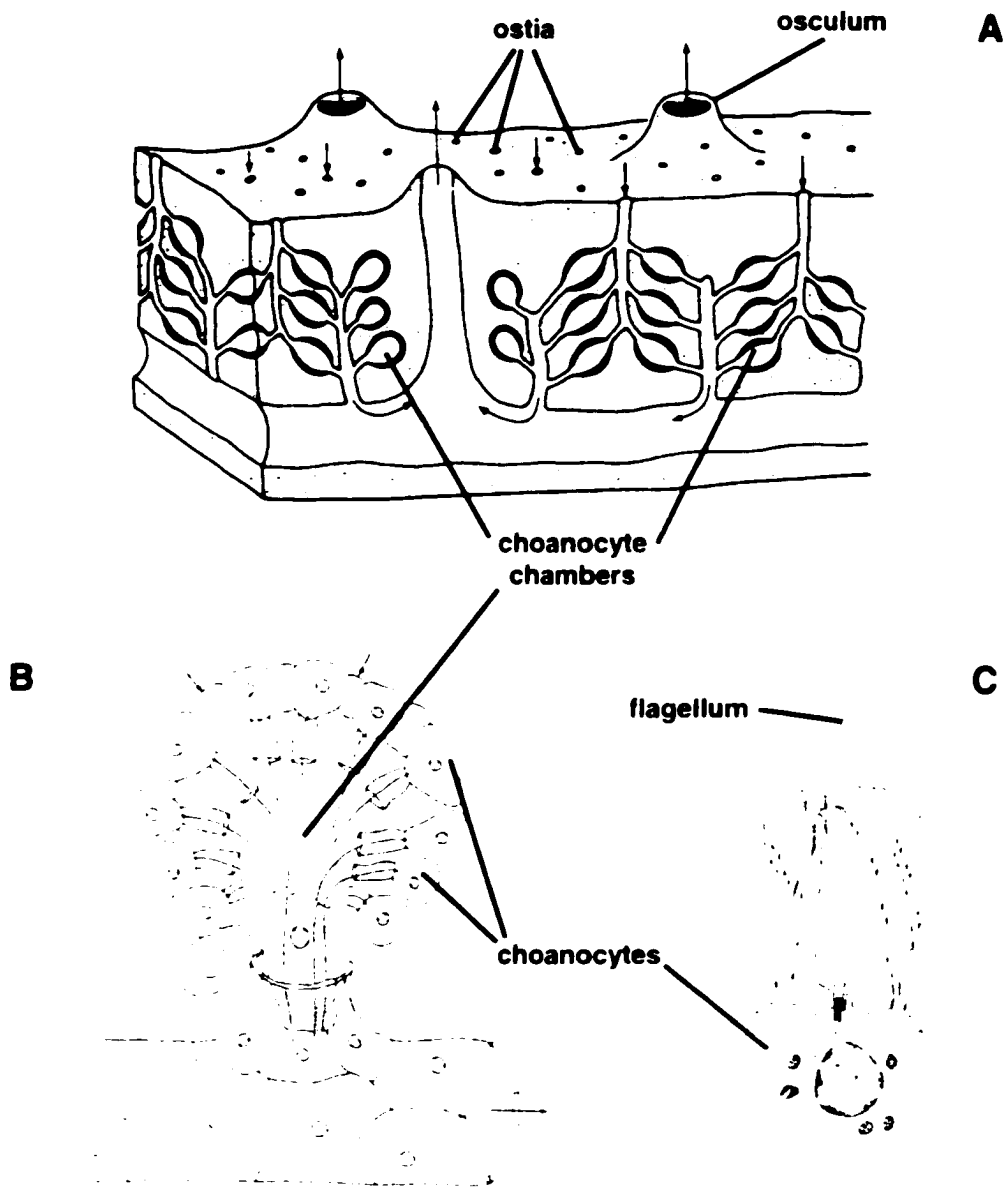

Figure 1.2: Structural design of an encrusting sponge. A. Aquifer system. B. Choanocyte chamber. C. Choanocyte cell. Small arrows indicate direction of water flow. Diagrams modified from Kozloff (1990).

Sponges can reproduce sexually or asexually (Fig. 1.3; reviews in Berquist et al., 1970; Simpson 1980). When reproducing sexually, mature sponge colonies will typically produce one type of gamete at a time, either sperm or eggs. The male gametes are released into the water column and filtered from the water by the choanocytes of another reproducing sponge. The sperm is transferred to fertilize the retained oocytes. Embryonic and early larval development often occur within the parent colony. After a period of growth and development, the larvae are released into the water column for a short planktonic phase. The larvae then recruit and settle to the benthos and upon locating a suitable habitat will grow and mature. Asexual modes of reproduction are often as important as sexual means for sponge propagation. The most common form of asexual reproduction is budding or fragmentation where a portion of a mature colony is tom away. The fragment is carried in the water and can potentially settle to substrate, reattach, and continue growing and reproducing. Gemmule formation is another mode of asexual reproduction, more common with freshwater sponges than marine sponges. An over-wintering cyst of cells is produced by a mature colony that is unable to survive extreme environmental conditions. When conditions more favorable for sponge growth return, the cyst is activated and a new sponge colony begins growing.

In marine communities sponges typically are not dominant space occupiers. Exceptions have been found (e.g., Dayton et al., 1974), but not frequently in the intertidal zone. When sponges do occupy large amounts of

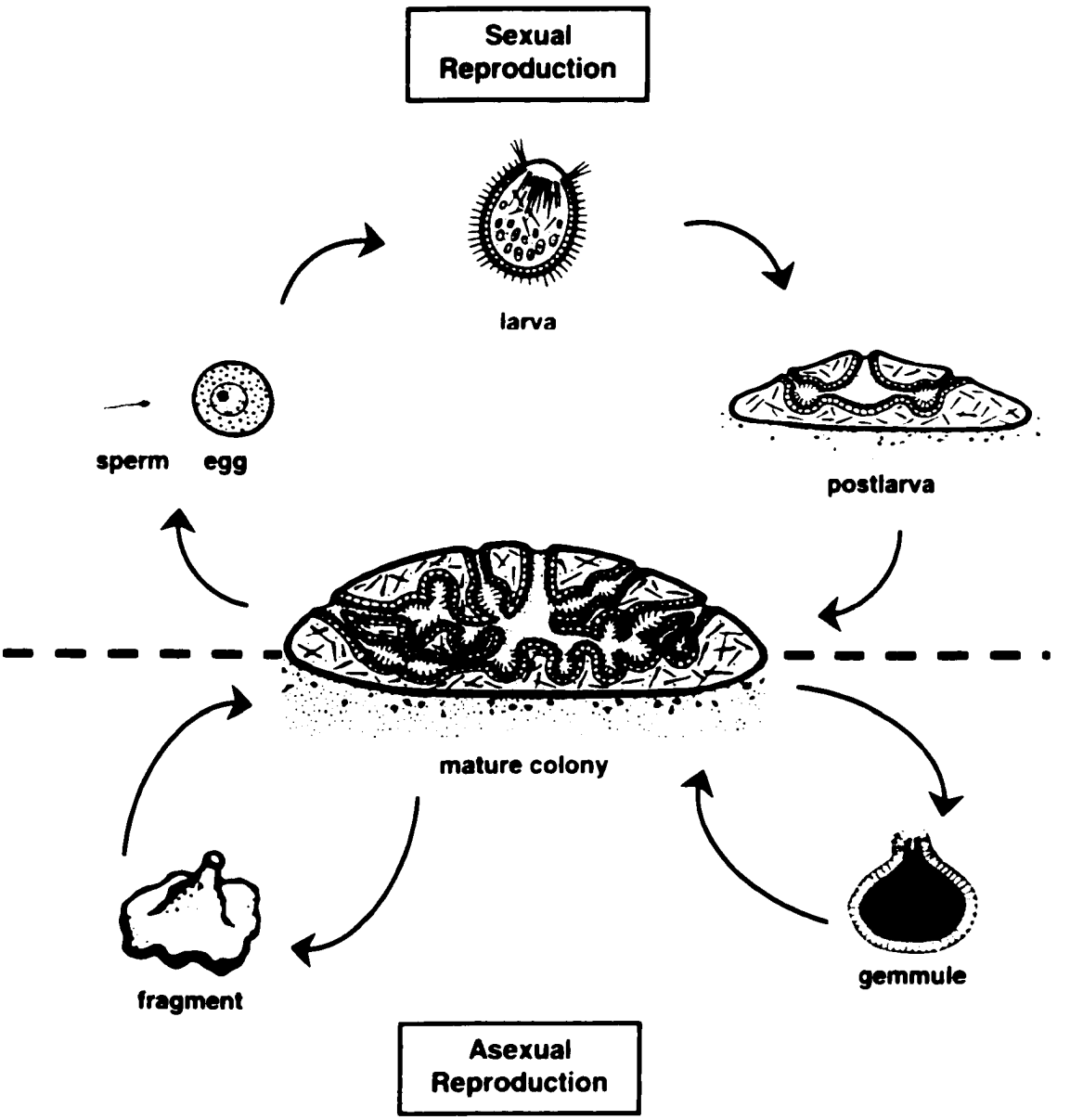

Figure 1.3: Sponge life cycle. Diagrams modified from Pearse et al., (1987).

primary space within an intertidal community, as seen at several locations in Kachemak Bay, experimental manipulations are more feasible. This study was initiated to investigate species interactions within sponge-dominated intertidal communities and to determine relevant ecological and biological mechanisms that allowed sponges to become spatially dominant.

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Chapter 2: Structuring Mechanisms in a Sponge-Dominated Intertidal Community

Introduction

A goal of ecological investigations is to understand how communities function. To attain this goal researchers focus on interactions occurring within subsets of the total species composition to determine driving mechanisms that shape the communities. Classical manipulative experiments demonstrated the potential importance of resource competition and predation in structuring temperate rocky-intertidal communities (Connell 1961, 1970; Paine 1966, 1974; Dayton 1971). While these results have been shown to be consistent within a defined set of conditions, a change in parameters such as larval recruitment success (Roughgarden et al., 1986; Underwood and Fairweather 1989; Carroll 1996; Connolly and Roughgarden 1999), latitudinal variation in species composition, species near the extreme ranges of their geographical distribution, or differences in local primary productivity, can produce different responses than predicted by the classical paradigms.

To investigate potential structuring mechanisms in a high-latitude, sponge-dominated rocky intertidal community, experiments on space competition, predation, and impact of partial predation (grazing) were conducted. The first

experiment examined the effect of overlying macroalgae on an established community of the sponge *Halichondria panicea.*

A second experiment studied *Halichondria panicea*'s primary predator, the dorid nudibranch *Archidoris montereyensis* and the effect prey quality had on its feeding, growth, and egg production. *H. panicea* often contains symbiotic zoochlorellae, unicellular green algae. The retention of zoochlorellae in sponge tissue may alter the nutritional value of the sponge to *A. montereyensis.* Two contrasting hypotheses were of interest. First, sponge containing symbiotic algae would be a better food source for the nudibranchs by the addition of nutrients contained within the algal cells. On the other hand, zoochlorellae could be a deterrent to *Archidoris* predation or reduce growth rates due to toxic substances potentially produced by the algae. The null hypothesis would be that there is no difference in the nutritional quality of *Halichondria* containing or devoid of symbiotic zoochlorellae. *A. montereyesis'* response to the presence or absence of symbiotic algae in their diet was examined under laboratory conditions.

The final experiment focused on the response of *Halichondria panicea* to a simulated predation event. The feeding activity of *Archidoris montereyensis* creates grooves and tunnels in the sponge colonies. The ability of sponge colonies to recover from predation events contributes to the persistence of a sponge population at a particular location. In order to evaluate sponge recovery responses, an artificial predation event was created by removing portions of

sponge colonies similar in size and shape to a nudibranch feeding scar. Sponge colony responses and recovery rates were observed and documented.

Materials and Methods

Study Site

The study was conducted at Outside Beach Park, Seldovia, Alaska, near the University of Alaska's Kasitsna Bay Laboratory on the Kenai Peninsula (Fig. 2.1). The region is highly productive due to upwelled water from the Gulf of Alaska entering Kachemak Bay (Sambrotto and Lorenzen 1986). Strong tidal currents resulting from an extreme tidal range of about 8 meters distribute nutrients and food. The study site is a horizontal section of semi-exposed, mid-level rocky intertidal beach approximately 55 m X 10 m. The beach consists of many large boulders intermingled with gravel and bedrock, with the sponge *Halichondria panicea* covering upper rock surfaces. The boulders are sufficiently large and embedded in the gravel that they approximate the stability of bedrock on ecological time scales. A diverse and abundant macroalgal assemblage is present along the shore consisting of bands of green, red, and brown species parallel to the water line (Dames & Moore 1977). The most abundant algal species present within the sponge zone during the summer is the annual brown alga *Alaria marginata* which blankets boulders and bedrock at low tide. Other prominent but smaller-sized algae include species of *Ulva*, *Halosaccion*, and

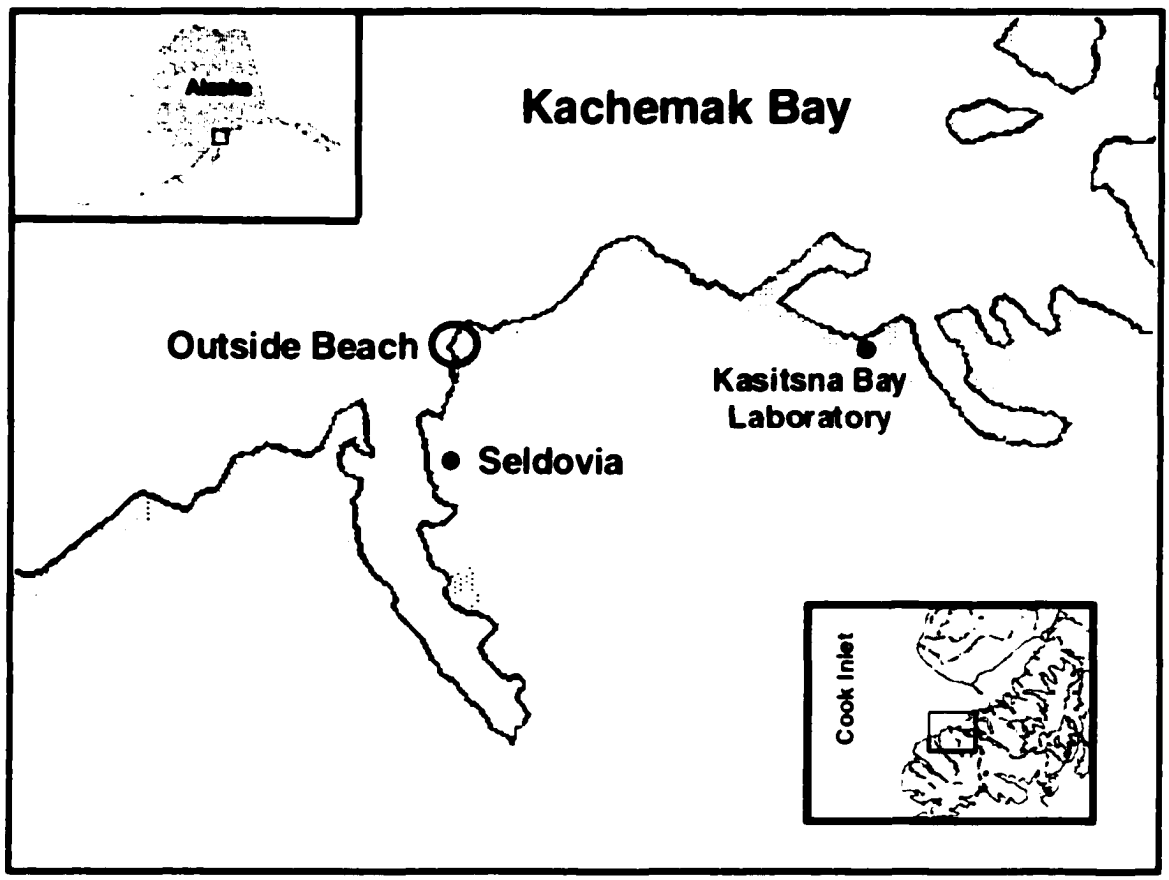

Figure 2.1: Location of study site at Outside Beach Park, near Seldovia, Alaska. Insets show regional context.

Palmaria. Few barnacles, primarily *Semibalanus cariosus*, were present within the sponge zone. Mussels were absent.

Space Occupation

Upper canopy composition and primary space utilization were measured from August 1994 through August 1996 in ten permanent 0.25 m^2 quadrats (Fig. 2.2A, B). Locations of the quadrats were haphazardly chosen within the sponge zone by blind tosses of a marker over the shoulder. Areal coverage was estimated monthly April through August and bimonthly September through March using a 0.25 m^2 quadrat frame with a string grid having 81 intersections. Point counts were recorded for each intersection to estimate species composition of components of the upper canopy layer and of the primary space occupiers attached to rock surfaces. Data were taken in four categories, *Halichondria panicea*, macroalgae, other organisms, and open rock. A photographic record of the permanent quadrats and of the site in general was compiled for most sampling dates.

To investigate the influence of algal canopy on *Halichondria panicea* abundance, half of the permanent quadrats were randomly chosen for removal of overlying macroalgae, while the remainder were undisturbed. Algae were removed in July 1994 and monthly April-July 1995. After July 1995, macroalgae were no longer removed and all flora were allowed to grow without further intervention. Percent cover data collected for both upper canopy layer and

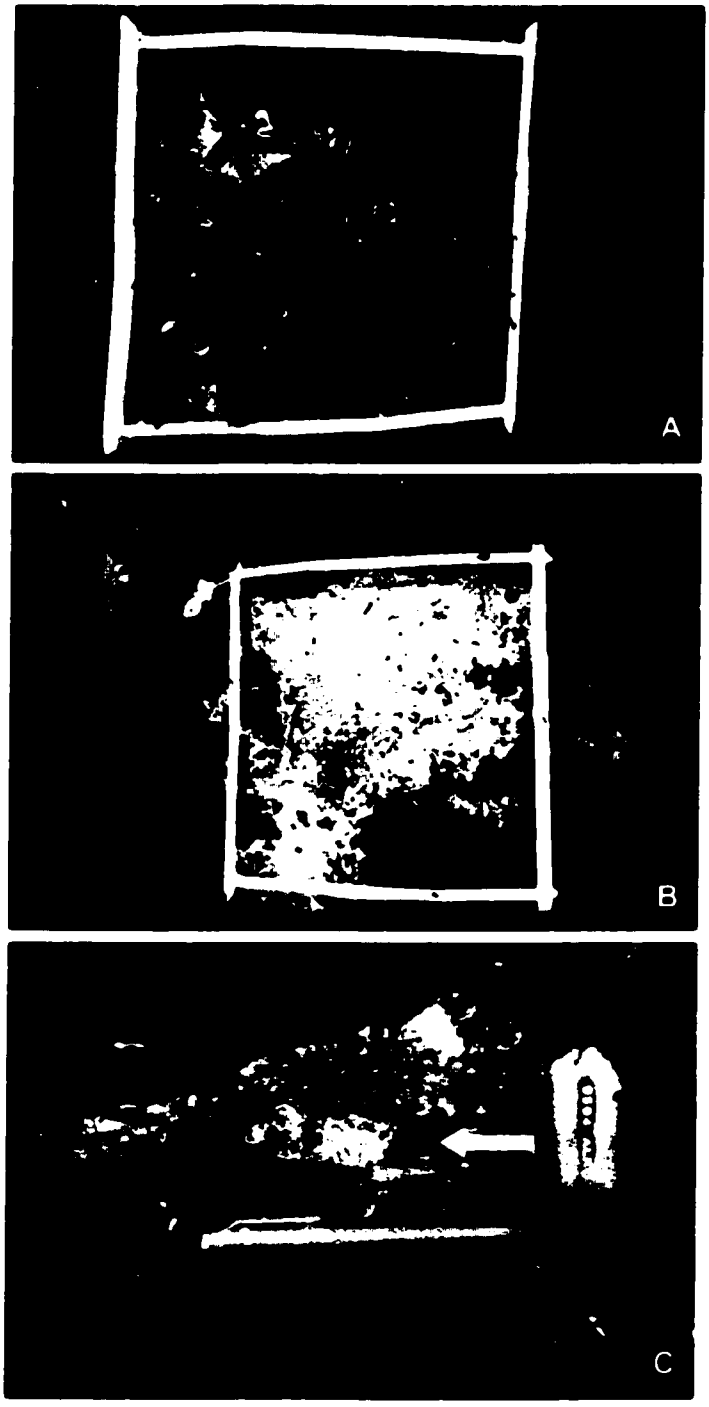

Figure 2.2: Photographs of experimental manipulations of sponge colonies. A. One of five permanent quadrats where the macroalgal canopy was not removed. B. Permanent quadrat after removal of algal canopy. C. Experimental "feeding" groove (arrow) cut into a sponge colony.

primary space utilization from August 1994 through August 1996 were analyzed for trends of treatment effects by comparing the slopes of regression lines between treatment and control quadrats for each data category with a two-sample t-test and the Wilcoxon rank sum test ($\alpha = 0.05$ for both).

Nudibranch Feeding Trials

In order to assess and quantify the relationship between *Halichondria panicea* and *Archidoris montereyensis*, a laboratory feeding trial was performed during summer 1995. Twenty-two pairs of size-matched *A. montereyensis* were placed in separate flow-through aquaria. As nudibranchs are cross-fertilizing hermaphrodites, they were paired to simulate field conditions. For data analyses, each nudibranch pair was treated as a single entity by combining their weights, sponge consumption and egg production. Nudibranchs for this experiment were collected from various intertidal beaches near the Kasitsna Bay Laboratory, but not from study sites established for other aspects of this research. Tanks were randomly assigned one of two different foods making 11 nudibranch pairs per treatment. Half of the nudibranchs were fed *H. panicea* with zoochlorellae and the other half were fed aposymbiotic *H. panicea*. Zoochlorellae were removed from the latter sponge tissues by maintaining the sponges for 2-3 weeks in seawater tanks completely covered with black plastic to block sunlight (Frost and Williamson 1980). Without light, the green coloration of the sponges

disappeared and it was assumed that the zoochlorellae were no longer present in the sponge tissues, though not confirmed histologically.

To facilitate use of body length as an index of wet-weight biomass, 46 *Archidoris montereyensis* were collected and regression curves fitted to the weight-length measurements. Each nudibranch was transferred to a shallow container filled with fresh seawater and allowed to relax before measuring its length to the nearest millimeter. Individuals were then removed from the water, lightly patted dry on a paper towel and weighed.

For *Halichondria panicea* measurements, water displacement volume and wet weight of 84 variously sized portions of freshly collected sponge tissue were determined. All visible rocks and shells embedded in the sponge tissue were removed to prevent measurements from being skewed. Each portion was quickly transferred from holding tanks to a graduated cylinder partially filled with fresh seawater and displacement volume was measured to the nearest milliliter. The sponge was removed from the cylinder, allowed to drain briefly, and weighed.

The feeding trials ran for 21 days. An ample supply of sponge was maintained in each nudibranch tank and the volume of sponge added at any time recorded. Weights and lengths of each nudibranch were measured at the start of the experiment and again at weekly intervals. During the course of the feeding trial, freshly laid egg ribbons were removed from the tanks, weighed, and recorded. At the completion of the experiment, final weights and lengths of the

nudibranchs were measured and the volume of sponge remaining in each tank recorded. Data were analyzed for effect of food type, as well as to determine rates of sponge consumption, egg ribbon production, and growth by the nudibranchs. Qualitative independent variables, new independent variables (indicator variables) created from categorical variables, were used in order to compare the slopes of the regressions of both groups (Ostle and Malone 1988). Significance was determined at the $\alpha = 0.05$ level.

Sponge Recovery Rates

During the summer of 1994, ten artificial feeding grooves were established in separate sponge colonies by removing a 2 cm x 10 cm portion of the colony down to the underlying substrate (Fig. 2.2C). Ten additional grooves were established and monitored in 1995. A photographic record of each artificial groove was made at 2-4 week intervals until the sponge reclaimed the groove space or up to a maximum of four months. Artificial grooves created in 1994 were monitored July-October; 1995 grooves, May-August. The photographs were digitally scanned and areal coverage of sponge within the artificial groove was measured using a digitizing tablet and image analysis software (SigmaScanPro). One groove for each year was removed from the analyses due to poor quality photographs. An additional groove was removed from 1994 analyses due to unexplained enlargement of the groove area as determined by changes in groove shape from photographs. Recovery growth rates for each

experimental colony were estimated using the area of substrate reclaimed between observation dates.

Results

Space Occupation

In addition to *Halichondria panicea*, species that were identified and counted as part of the community included many species of fleshy algae, two types of coralline algae, and several mobile or sessile benthic invertebrates (Table 2.1). Coralline algae were included in the 'other organisms' category because they were readily overgrown by *Halichondria*, provided little shading to the sponge, and were not among the fleshy algal species that were removed from treatment quadrats. Field identifications were made to the lowest taxonomic level possible, but data analyses were conducted using the categories *Halichondria*, macroalgae, other organisms, and open rock.

The *Halichondria* and algal components of the upper canopy layer (Fig. 2.3A, B) exhibited greater seasonal changes than they did in the primary space analysis (Fig. 2.4A, B). These observations were driven by the annual life cycles of the macroalgae. While there were no significant differences in trends between treatment and control quadrats for other organisms and open rock (Fig. 2.3C. D), statistically significant differences were observed for both *Halichondria* and macroalgal composition of the upper canopy layer (Table 2.2). Removal of

Table 2.1: Macroalgae and other organisms catalogued in permanent quadrats, August 1994-August 1996. Listing is alphabetical.

Macroalgae	**Other Organisms**
Acrosiphonia sp.	*Archidoris montereyensis*
Alaria marginata	Barnacles
Cymatheria triplicata	Coralline algae, geniculate
Filamentous brown alga	Coralline algae, non-geniculate
Filamentous green alga	*Epiactis prolifera*
Laminaria bongardiana	*Henricia* spp.
Halosaccion sp.	*Katharina tunicata*
Iridea sp.	Limpets
Odonthalia sp.	*Mopalia* spp.
Palmaria spp.	Tunicata, colonial ascidian
Porphyra sp.	Unidentified white sponge
Tokita dendron	
Ulva sp.	

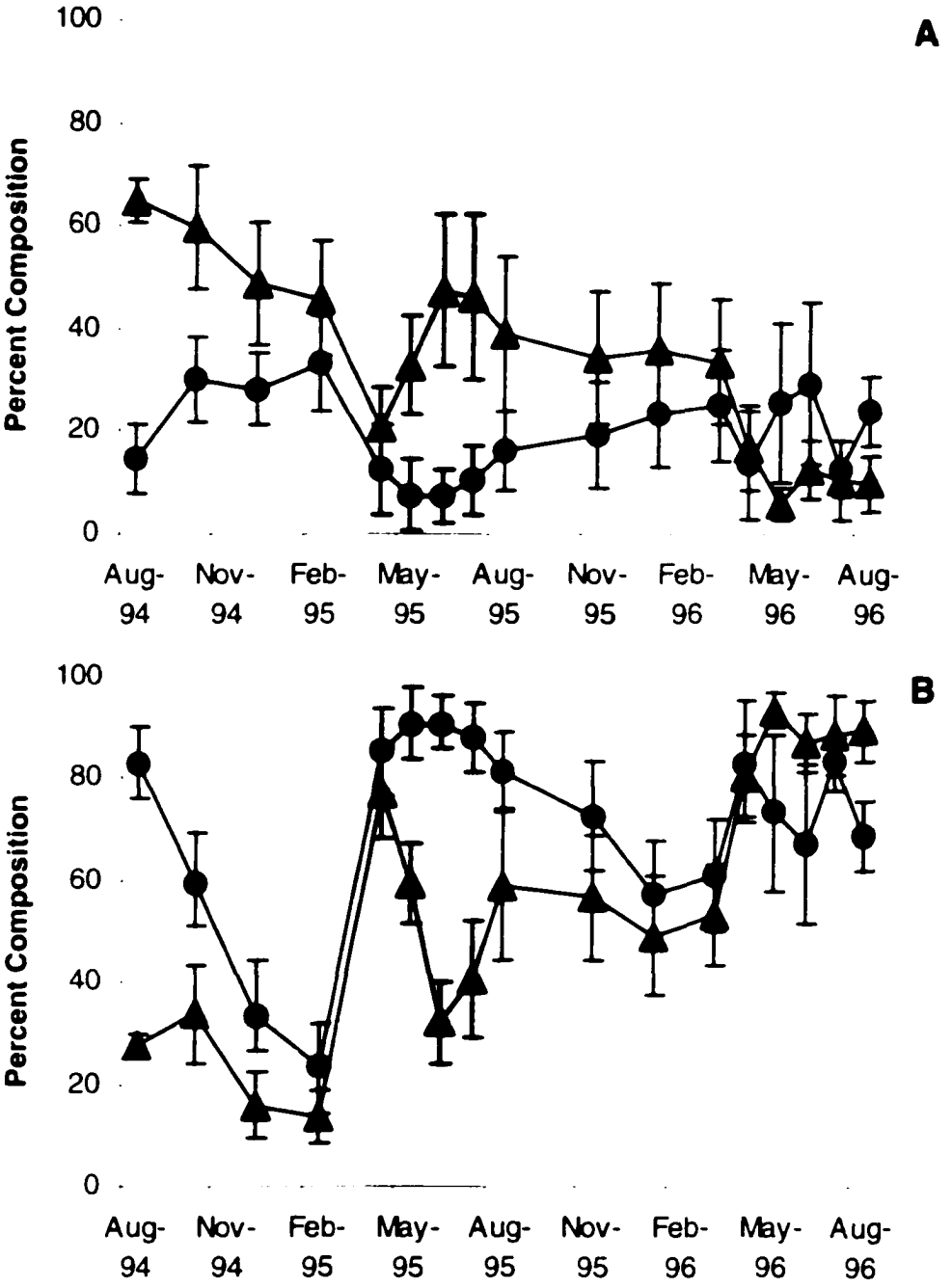

Figure 2.3: Percent composition of upper canopy layer for *Halichondria*, macroalgae, other organisms, and open rock in ten permanent quadrats, August 1994-August 1996. Non-clipped quadrats are circles (●, n = 5) and clipped quadrats are triangles (▲, n = 5). Shaded bar indicates when macroalgae were removed in 1995. Removals also occurred in July 1994. Mean ± 1 standard error. Sampling dates are along the x-axis. A. *Halichondria*. B. Macroalgae.

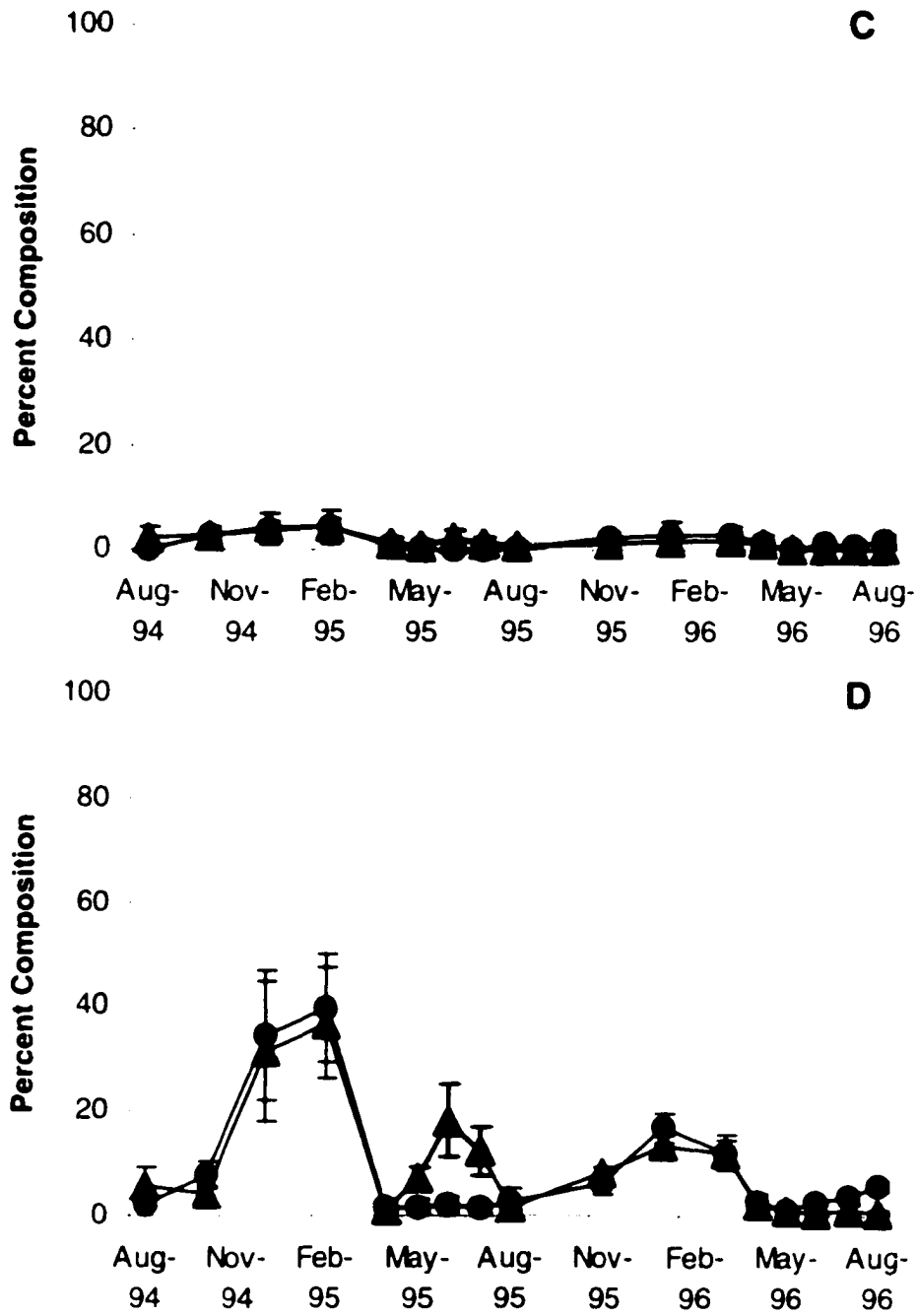

Figure 2.3 (continued): C. Other organisms. D. Open rock.

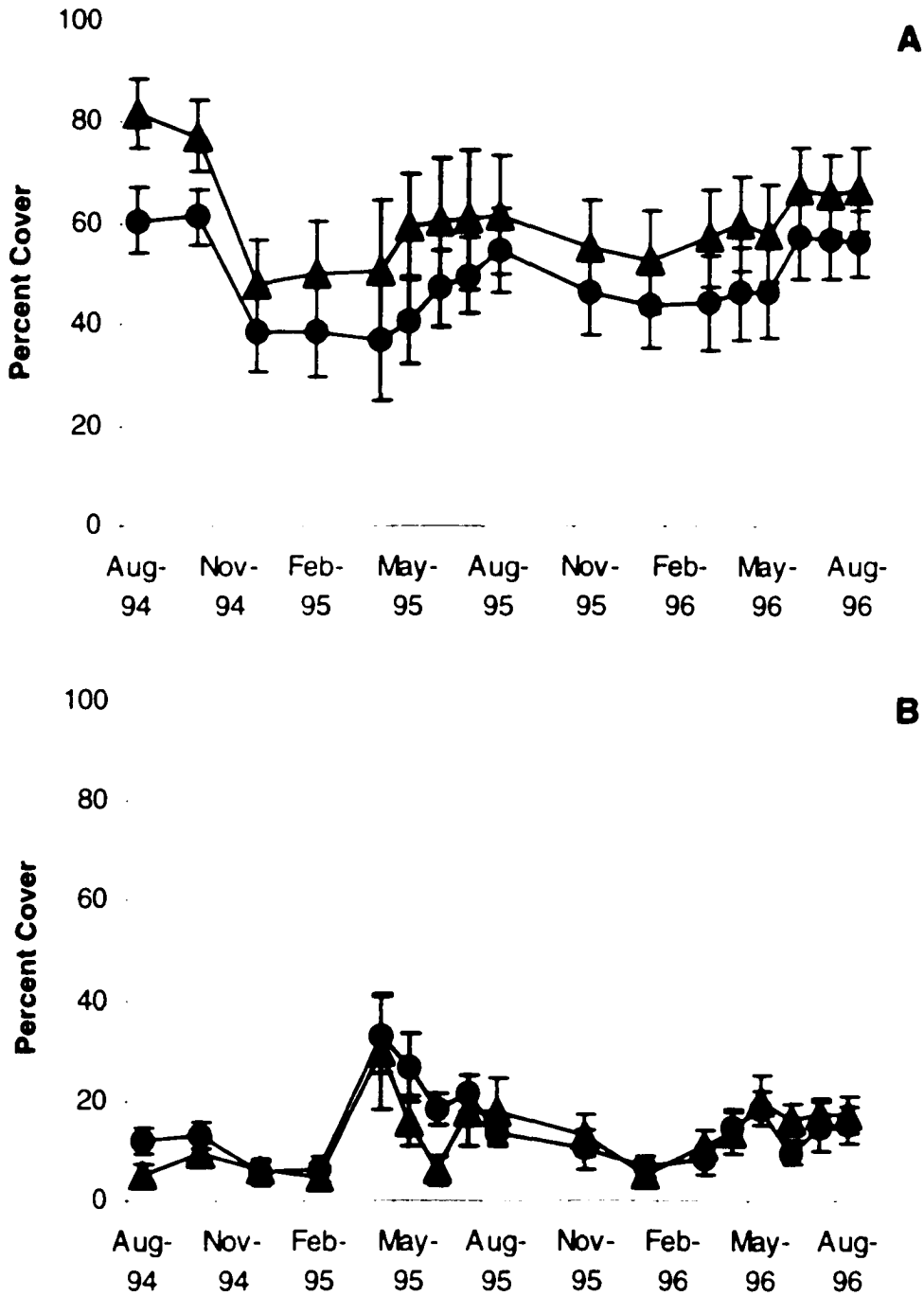

Figure 2.4: Percent rock surface area for *Halichondria*, macroalgae, other organisms, and open rock in ten permanent quadrats, August 1994-August 1996. Non-clipped quadrats are circles (●, n = 5) and clipped quadrats are triangles (▲, n = 5). Shaded bar indicates when macroalgae were removed in 1995. Removals also occurred in July 1994. Mean ± 1 standard error. Sampling dates are along the x-axis. A. *Halichondria*. B. Macroalgae.

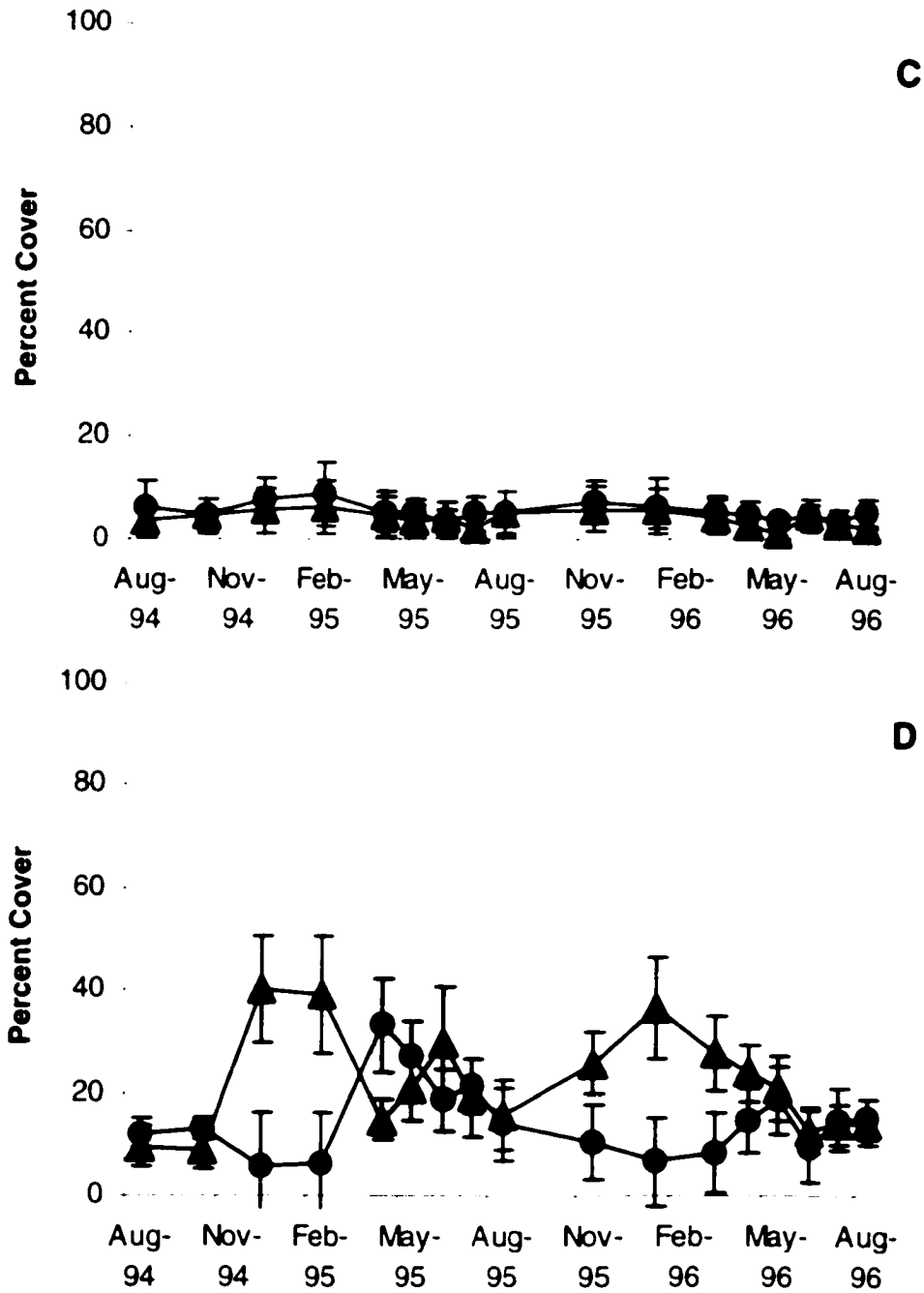

Figure 2.4 (continued): C. Other organisms. D. Open rock.

macroalgae occurred just prior to the first observations in 1994 and again during the summer of 1995. The manipulation is reflected in the graphs by a dramatic decrease of macroalgae and corresponding increase of sponge in the treatment quadrats. While there was no actual increase in *Halichondria* cover, algal removal resulted in more sponge being counted under the grid intersections for the upper canopy measurements. The differences between *Halichondria* and algal cover decreased over time as the algae recovered.

Space utilization of the substrate surface remained relatively consistent over time between algal-removal and non-removal quadrats (Fig. 2.4A-C). *Halichondria panicea* (Fig. 2.4A) was the dominant space occupier throughout the study, covering 40%-80% of the rock surfaces. Apparent peaks for macroalgae (Fig. 2.4B) in the spring (March-May) and subsequent declines reflected annual cycles in abundance and growth of macroalgae, primarily *Alaria marginata*. For treatment quadrats, the decline of macroalgae in 1995 was enhanced by manual removal of macroalgae. Peaks in the percent of non-utilized rock surface of clipped quadrats (Fig. 2.3D) correspond with winter declines in both *Halichondria* and macroalgae. A similar pattern was observed for non-manipulated quadrats. Statistical analyses of primary space utilization trends between treatment and control quadrats for each category over the entire observation period, including one year post-algal removal, revealed no significant differences for *Halichondria* cover, other organisms, or open rock (Table 2.2). While not apparent from Figure 2.4B, a statistically significant difference was

Table 2.2: Results of two-sample t-tests on the slopes of regression lines for each category of space utilization from five treatment and five control quadrats, August 1994-August 1996. The same results were attained using the nonparametric Wilcoxon rank sum test. Significance level for both analyses was $\alpha = 0.05$. Asterisk (*) = significant difference, ns = not significant.

	Upper Canopy	Rock Surface
Halichondria	*	ns
Macroalgae	*	*
Other Organisms	ns	ns
Open Rock	ns	ns

H_o: slope of treatment quadrats = slope of control quadrats

H_a: slope of treatment quadrats ≠ slope of control quadrats

detected in the trends of macroalgal substrate utilization. Macroalgae in treatment quadrats had a slightly positive slope (=increasing) reflecting the increase of macroalgae in the quadrats after removals ceased, while macroalgae in the control quadrats held steady or slightly decreased.

Nudibranch Feeding Trials

Log nudibranch wet weight and log length measurements were highly correlated (Fig 2.5A, $r^2 = 0.96$), implying a close, positive relationship between wet weight and length. The correlation in the log scale shows that length is a good surrogate for weight, so body length measurements were used to test for biomass changes during the experiment. For sponge tissue, water displacement volume and wet weight were also highly correlated (Fig 2.5B, $r^2 = 0.95$). These data provide a simple means of converting sponge volume fed to the nudibranchs during the experiment to grams of wet sponge tissue, a more useful unit of food consumption for comparative purposes.

A significant positive treatment effect was found for *Archidoris montereyensis* feeding, egg ribbon production, and growth rates when fed symbiotic *Halichondria panicea* (Table 2.3). Nudibranch feeding rate (average daily sponge consumption) was significantly correlated with initial weight (Fig. 2.6A). Average daily egg ribbon production rates vs. initial weight were also highly correlated (Fig. 2.6B). Larger nudibranchs tended to feed more and to produce more egg ribbons than smaller nudibranchs. The overall (all nudibranch

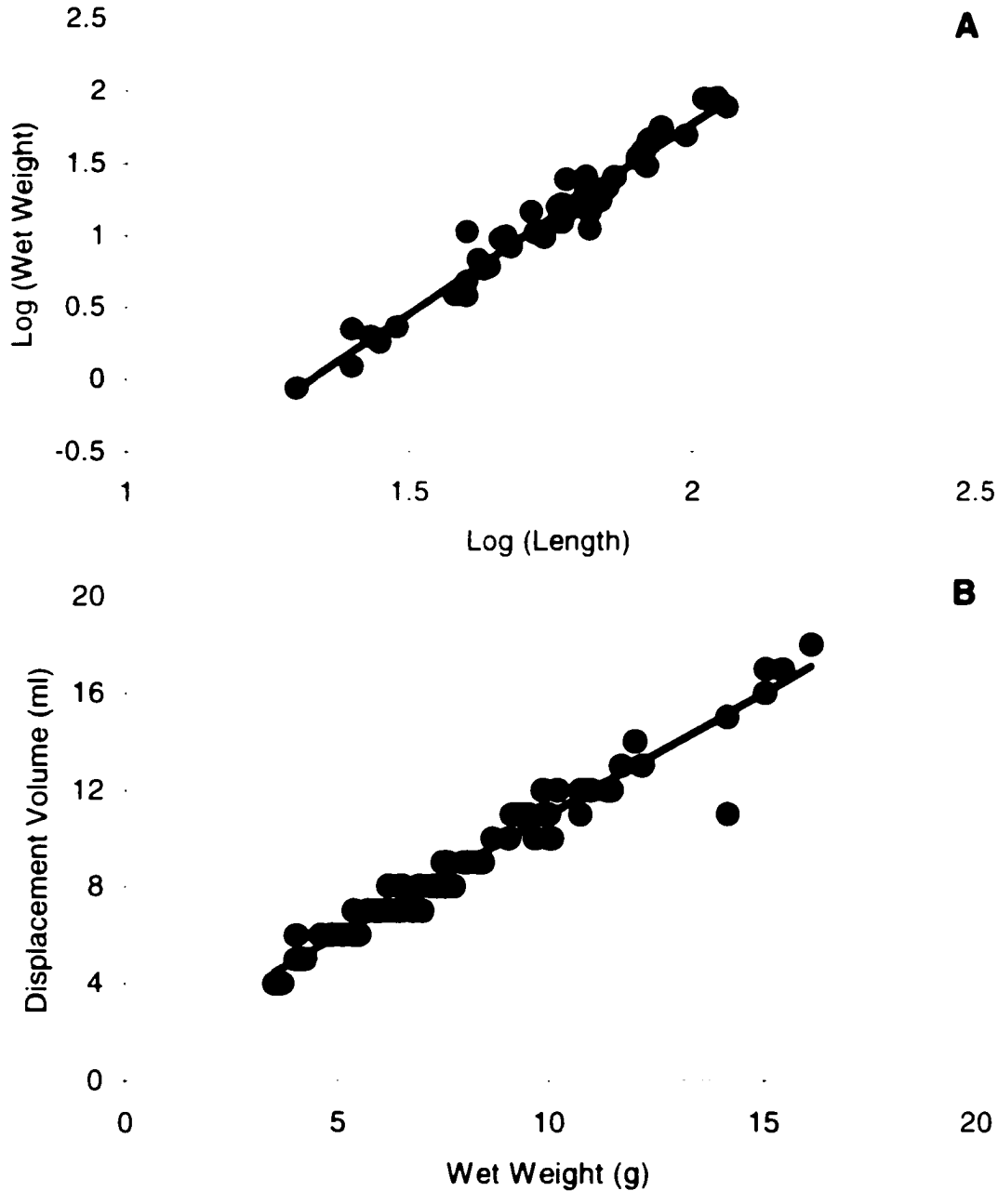

Figure 2.5: Linear relationship of size measurements for the nudibranch *Archidoris montereyensis* and the sponge *Halichondria panicea*. A. Wet weight vs. length relationship for *A. montereyensis*. Data were log-transformed for a linear model fit (r^2 = 0.96, n= 46). B. Displacement volume vs. wet weight relationship for *H. panicea* (r^2 = 0.95, n = 84).

Table 2.3: Summary of results for *Archidoris montereyensis* fed symbiotic and aposymbiotic *Halichondria panicea*. Values reported are the mean response and one standard error (in parentheses). Statistical analyses are based on weight-corrected regression comparisons. Asterisk (*) = significant difference, na = not statistically analyzed. N = 22, 11/treatment

	Sample Size Per Treatment	Nudibranchs Fed Symbiotic Sponge	Nudibranchs Fed Aposymbiotic Sponge	Regression Analysis ($\alpha = 0.05$)
Feeding Rate[1]	11	0.15 (0.02)	0.14 (0.03)	*[4] $p = 0.0002$
Egg Ribbon Production[2]				
All pairs	11	3.6×10^{-3} (9.7×10^{-4})	3.0×10^{-3} (8.9×10^{-4})	* $p < 0.0001$
Pairs laying ribbons	7	5.6×10^{-3} (7.6×10^{-4})	4.8×10^{-3} (8.3×10^{-4})	na
Growth Rate[3]				
All pairs	11	1.06 (0.16)	0.71 (0.10)	na
Pairs ≤60 g	9	1.08 (0.19)	0.72 (0.13)	* $p = 0.0001$
Pairs >60 g	2	0.96 (0.24)	0.66 (0.08)	na[5]

[1]Units: g sponge / g nudibranch • day
[2]Units: g egg ribbon / g nudibranch • day
[3]Units: g nudibranch / day
[4]Analysis based on log transformed data
[5]Sample size too small for statistical comparison

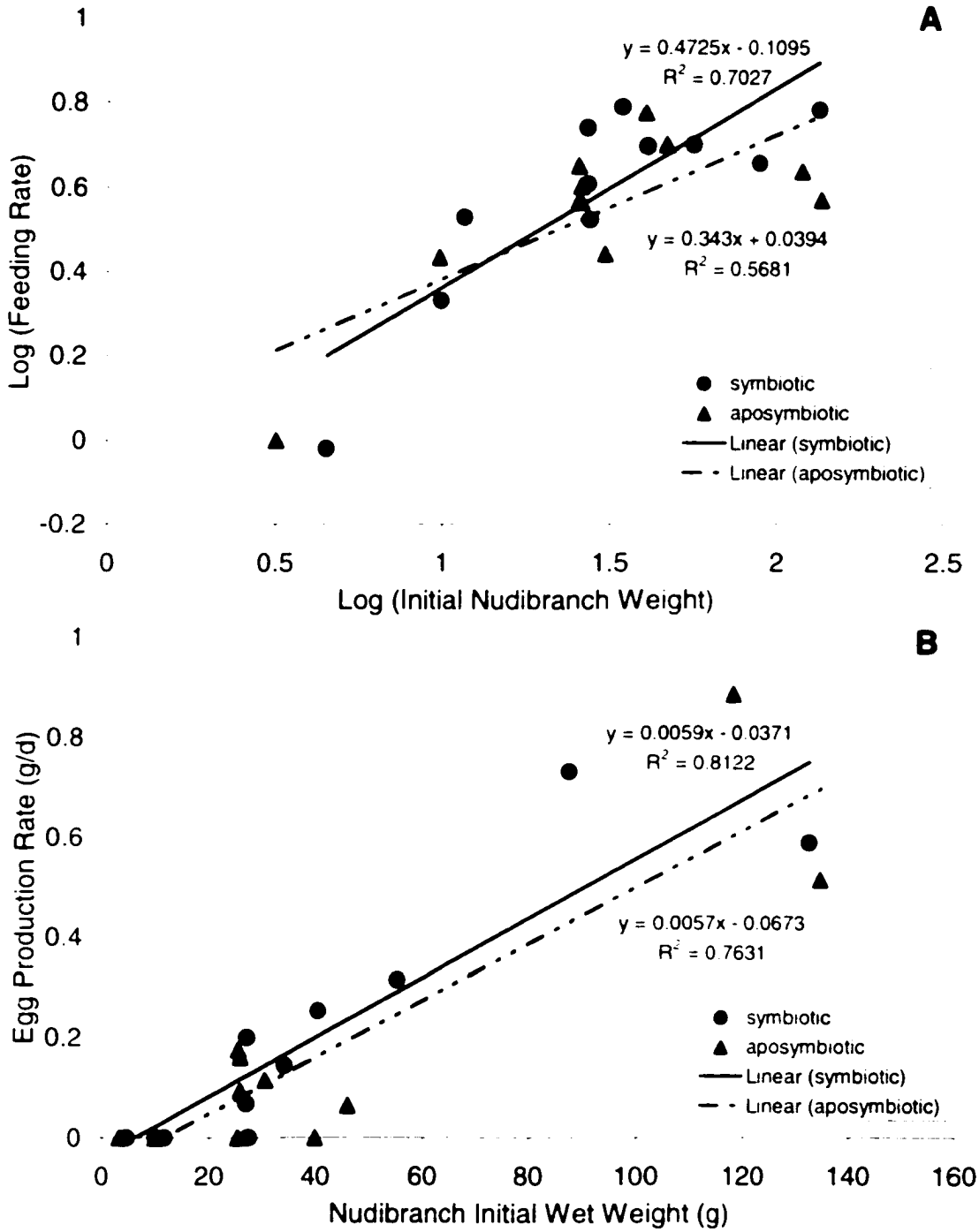

Figure 2.6: Feeding rates and egg ribbon production of *Archidoris montereyensis* fed symbiotic and aposymbiotic *Halichondria panicea*. A. Feeding rates relative to initial combined wet weight of each nudibranch pair. Data were log-transformed for a linear model fit. B. Egg ribbon production rates.

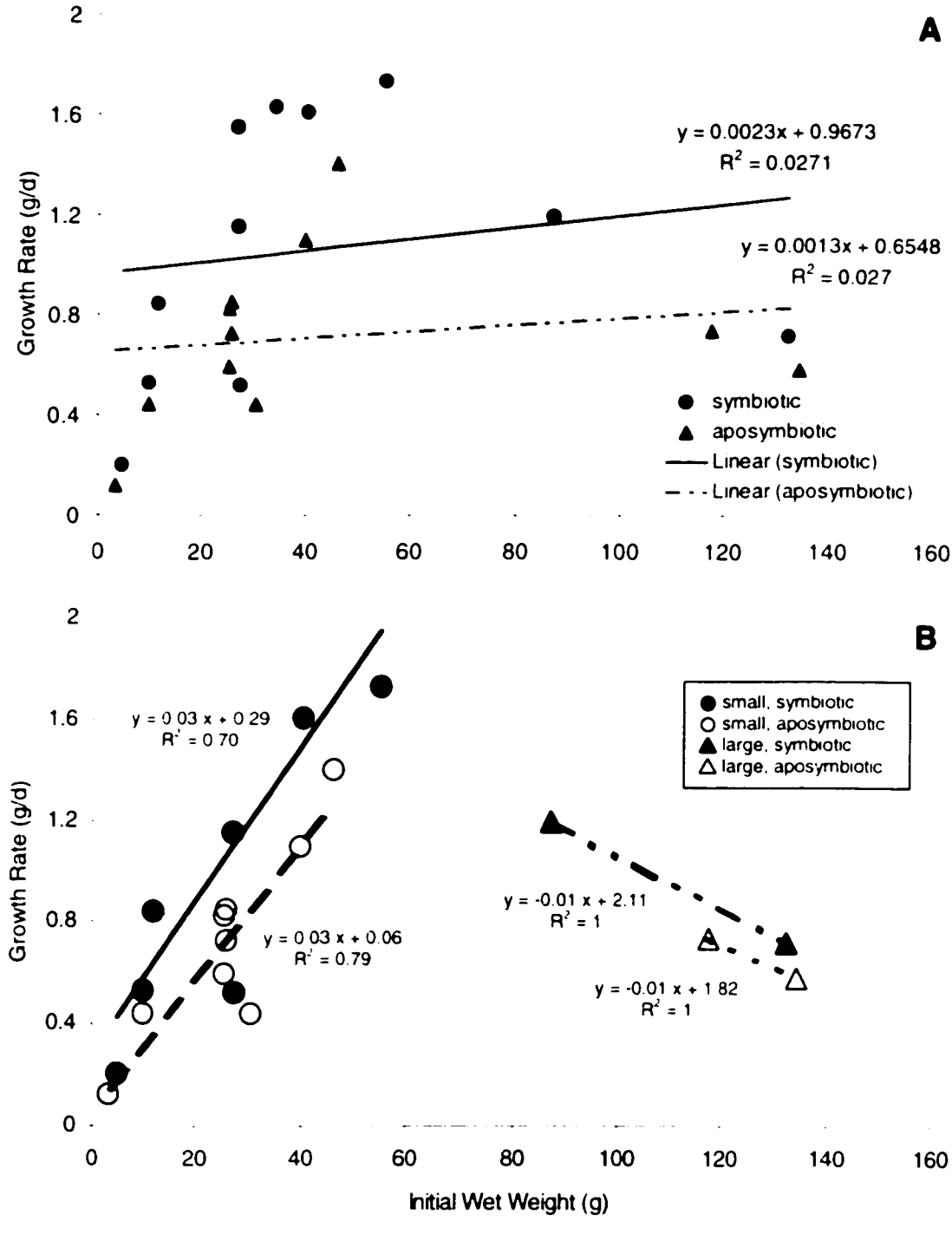

Figure 2.7: Growth rates of *Archidoris montereyensis* fed symbiotic and aposymbiotic *Halichondria panicea*. A. By treatment. B. Growth rate trends of small (≤60 g, circles) and large (>60 g, triangles) nudibranchs.

pairs included) regressions for growth rate (Fig. 2.7A) were weak due to a size-dependent shift in growth trends. A significant difference in growth rate trends was observed between large (> 60 g) and small (≤ 60 g) nudibranch pairs (t-test of slopes, $\alpha = 0.05$, Fig. 2.7B), regardless of treatment. Growth rates increased with increasing size for small nudibranchs but large nudibranchs tended to grow at a slower rate as they became larger, possibly due to increased egg production rates with size (Fig. 2.6B). It appears that more energy was allocated for reproductive output as nudibranchs reached a large size, decreasing the relative amount of energy available for somatic growth.

At the study site *Archidoris montereyensis* will most often encounter symbiotic *Halichondria panicea* (Knowlton *pers. obs.*). Based on experimental results for nudibranchs fed symbiotic sponge tissue, each *A. montereyensis*, in a single day, consumes 0.15 g sponge tissue per gram body weight, produces 5.6 x 10^{-3} g egg ribbon per gram body weight, and gains 1.06 g in body weight (Table 2.3). For example, a 25 g nudibranch would be expected to eat 3.75 g sponge (15% of body weight), produce 0.14 g egg ribbon, and gain 1.08 g body weight per day.

The relationship between egg ribbon production and growth rate was plotted and no trend was apparent when all nudibranch pairs were included (Fig. 2.8A). When pairs not producing egg ribbons were removed from the plot, a trend was observed (Fig. 2.8B). Nudibranchs with high egg production rates tended to have low growth rates and those with high growth rates tended to

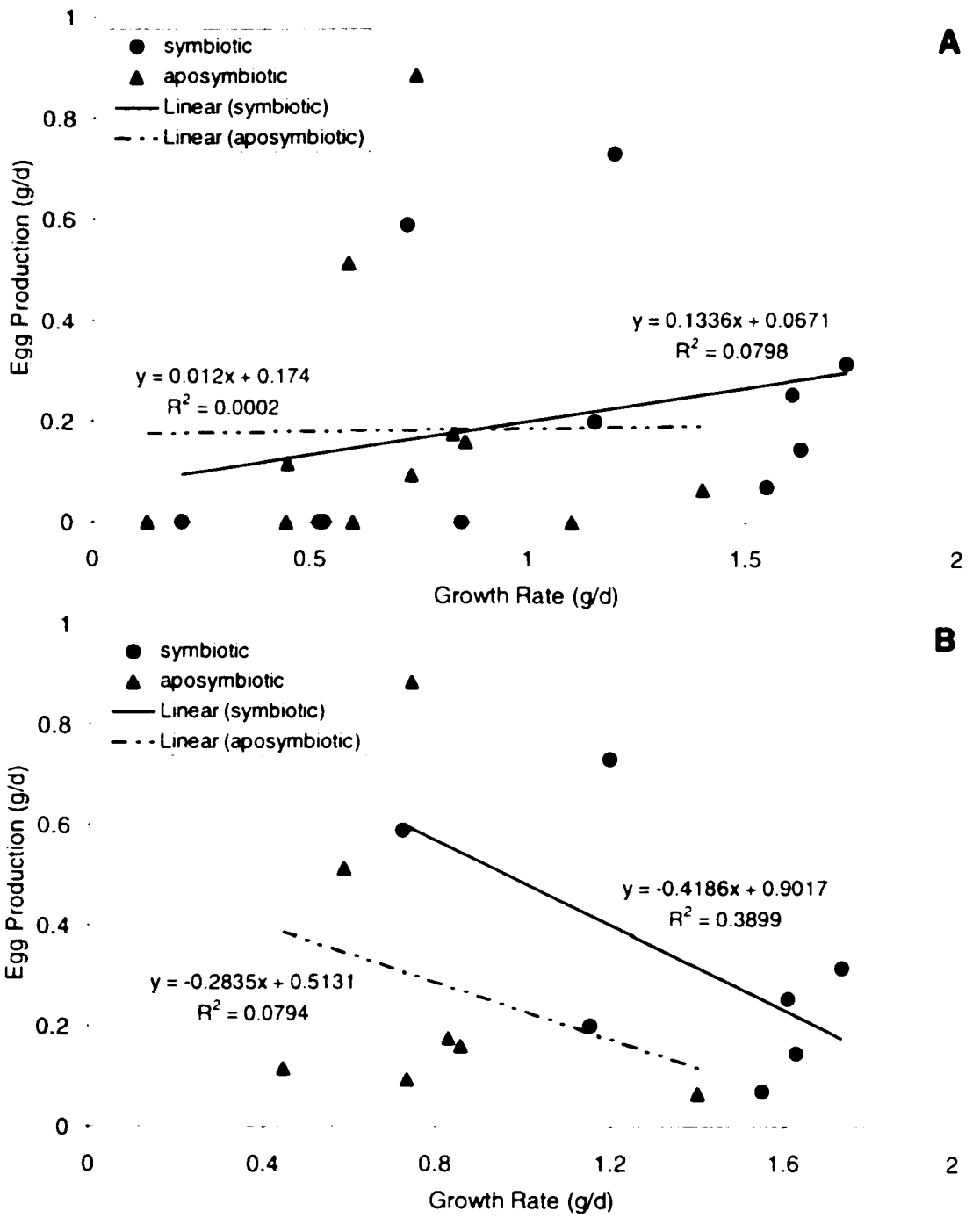

Figure 2.8: Relationship between egg production and growth rate of *Archidoris montereyensis* fed symbiotic and aposymbiotic *Halichondria panicea*. A. All nudibranch pairs in the study (n = 22). B. Nudibranch pairs actually producing egg ribbons (n = 18).

produce few egg ribbons, again indicating an energy trade-off between growth and reproduction rates.

Sponge Recovery Rates

Sponge colonies with artificial nudibranch feeding grooves recovered on average about 68% of the groove area within the first month or less (Fig. 2.9). Most colonies achieved 75%-100% recovery within the four month experimental period. Growth rates were estimated for time periods between observations and declined over time as the artificial grooves recovered (Fig. 2.10). During the first 4 weeks following the simulated predator event, growth rates were the highest, ranging from 0.2-1.4 cm^2/day. Subsequent growth rates slowed to -0.1 to 0.4 cm^2/day. If a colony completely reclaimed the scar area between observations, it was dropped from the growth rate calculations for that time period because it could not be determined how long prior to the observation the area had been filled. The rapid initial growth rates indicate an active response to injury.

Discussion

Space Occupation

No significant effect of macroalgal cover on *Halichondria panicea* abundance was found. Several factors may have contributed to this result. First, the buffer zone around the algal removal quadrats was not large enough.

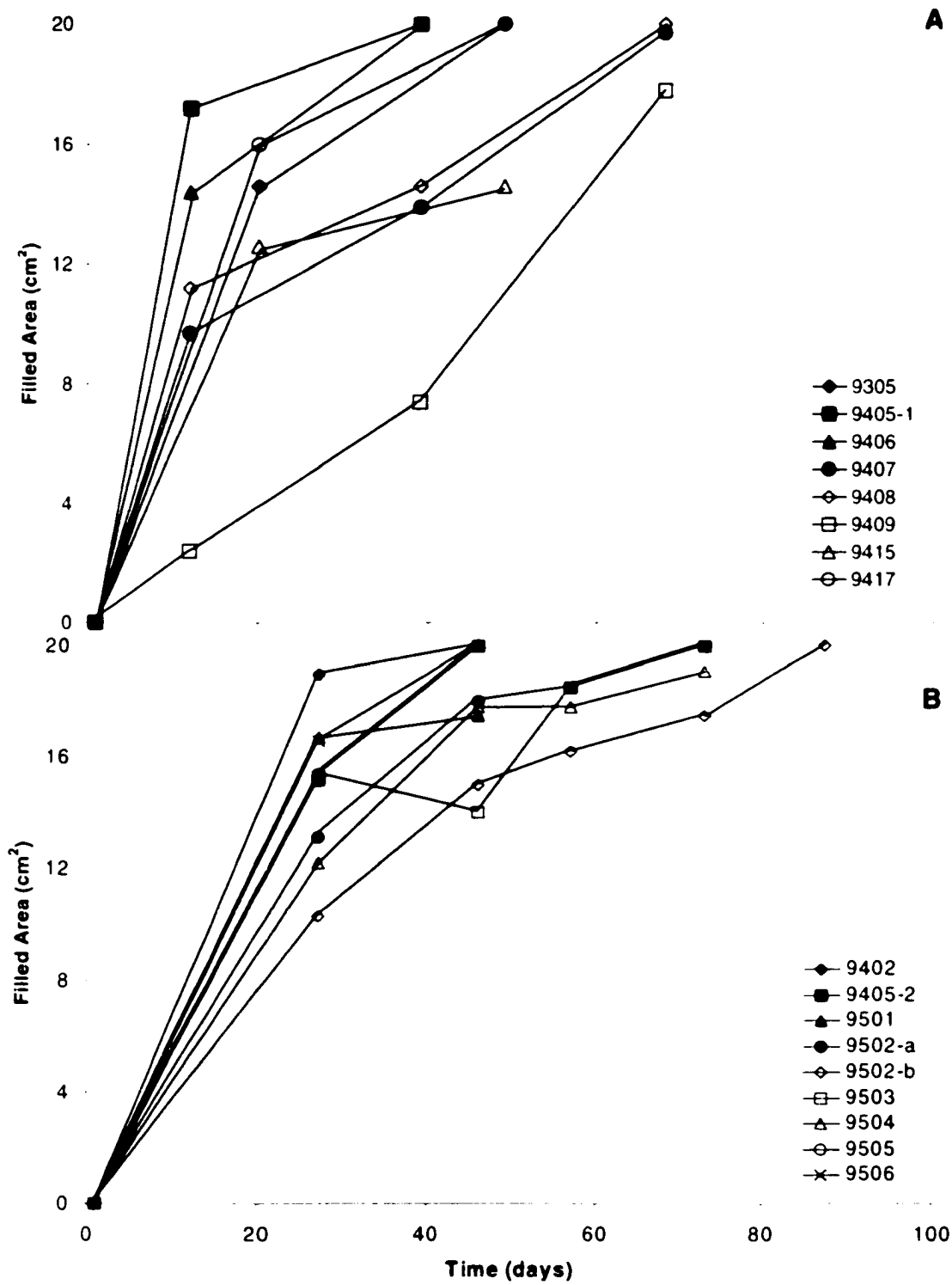

Figure 2.9: Growth of *Halichondria panicea* into artificial feeding groove areas cut into colonies. Each line represents a separate colony. A. 1994. B. 1995.

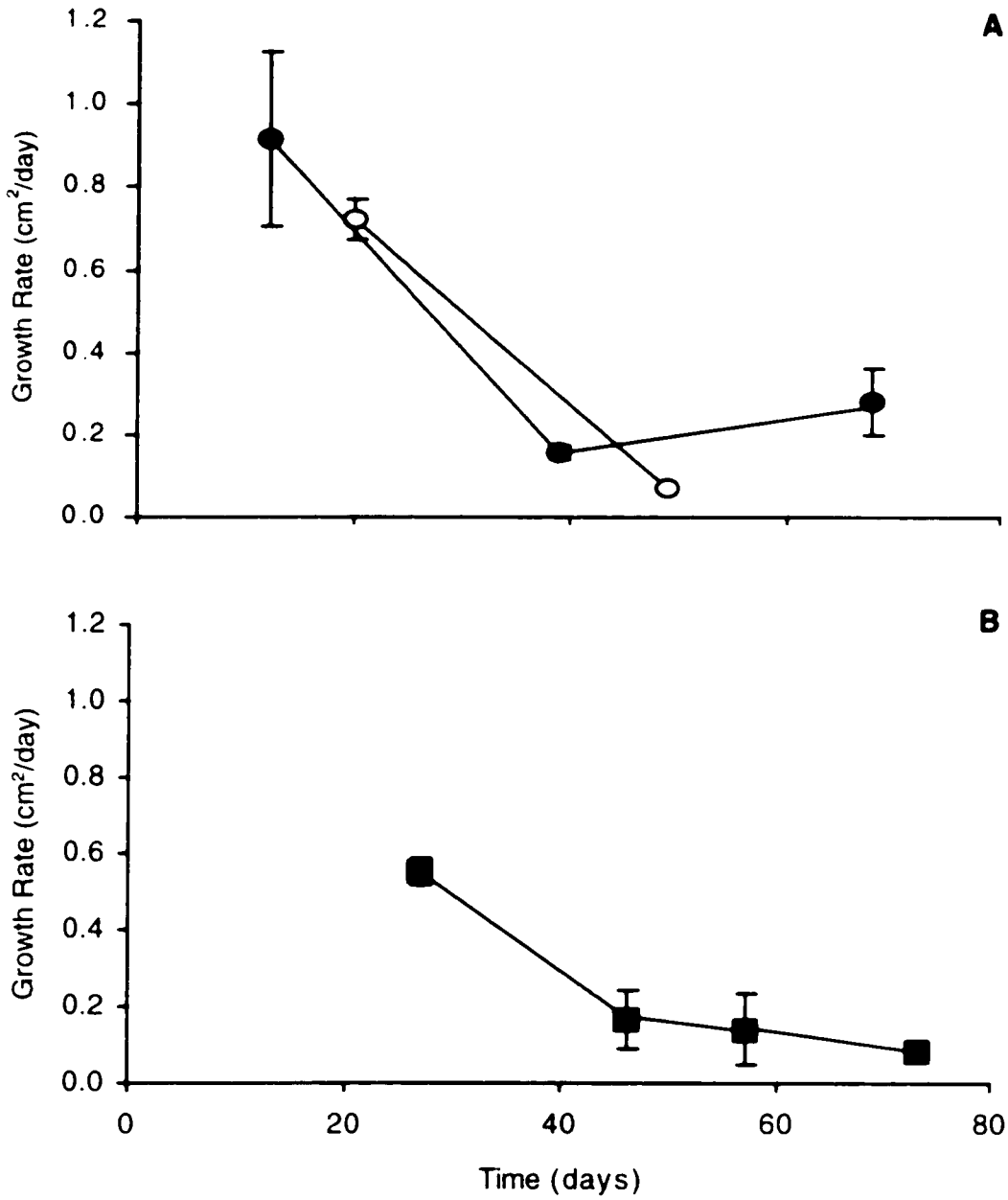

Figure 2.10: Estimated growth rates of *Halichondria panicea* into artificial feeding grooves. A. 1994. Group I (●); sample size for each growth period was n = 5, 3, and 2 respectively. Group II (○); n = 3 and 1 respectively. The groups were started at different times. Mean ± 1 standard error (SE). B. 1995 (■). Sample sizes were n = 9, 5, 4, and 2 respectively. Mean ± 1 SE.

Only a 0.5 m swath around each quadrat was also cleared of overlying macroalgae. The predominant macroalga on site was *Alaria marginata*, which can attain a blade length of up to three meters (O'Clair et al., 1996). Blades from adjacent areas were long enough to cover sponge located in treatment quadrats, thereby minimizing the differences between treatment and control quadrats during low-tide measurements.

Estimates of utilization of primary space have more relevance to long-term sponge population abundance investigations than canopy cover. Canopy cover is seasonally variable due to annual life cycles of predominant algal species. Direct measurements of sponge cover on the substratum provide a more accurate estimate of any changes in actual sponge abundance.

Relatively few studies have examined associations between sponges and macroalgae. Of those conducted, several indicate that certain macroalgal species depend on the co-occurrence of associated sponges without the sponges necessarily being dependent on the macroalgae (e.g. Scott and Wetherbee 1982; Price et al., 1984). Palumbi (1985) observed that the sponge *Halichondria panicea* was more abundant in the presence of foliose coralline algae than in its absence, but also noted that the relationship varied on a small spatial scale with no effect being detected in some instances.

There have been a few studies on interactions of sponges with other space occupiers. On coral reefs sponges have been described as dominant aggressors in the competition for space, overgrowing many species of corals, as

well as other sessile marine invertebrates, but also exhibiting a range of overgrowth capabilities among sponge species (e.g. Vicente 1990; Aerts 1998, 2000). Macroalgae can inhibit coral growth by contact interference with coral feeding activities and is capable of overgrowing coral colonies (e.g. Tanner 1995, Jompa and McCook 2002). Other factors such as herbivory, substrate stability, and growth rates, have been shown to mediate space competition in some cases for both sponges and macroalgae (Vicente 1990; Aerts 1998, 2000; Jompa and McCook 2002).

The results of this study do not clearly indicate whether or not *Halichondria panicea* and *Alaria marginata* competitively exclude each other from primary space utilization. Observations of sponge growing up and around *Alaria* holdfasts, as well as small algal sporlings growing out of sponge tissue (Knowlton *pers. obs.*), indicate that neither organism is totally restricted by the other. The presence or absence of a macroalgal canopy cover does not appear to influence the abundance of established *H. panicea*.

Nudibranch Feeding Trials

Archidoris montereyensis fed symbiotic *Halichondria panicea* exhibited greater feeding, egg ribbon production, and growth rates than did *A. montereyensis* fed aposymbiotic *H. panicea*. The presence of symbiotic zoochlorellae in the nudibranch's diet appears to be beneficial. Research on the symbiotic relationship between sponges and zoochlorellae indicates that

sponges benefit by receiving photosynthetic products, like reduced organic carbon (Muscatine et al., 1967) and sponge growth rates are enhanced when symbiotic algae are present (Frost and Williamson 1980). Presumably, nudibranchs could benefit from algal production, as well, by consuming photosynthate released into the sponge tissue and digesting algal cells. The presence of zoochlorellae in sponge tissue could simply increase the energy density of sponge tissue, providing the nudibranch with more nutrients per unit volume than sponge tissue without symbionts. On the other hand, sponges maintained under reduced light conditions may be negatively affected by the loss of photosynthate or deterioration of algal cells. Another possibility is that zoochlorellae utilize toxic or unpalatable chemicals, functionally removing them from the sponge tissue. The reduction or loss of zoochlorellae could allow the substances to build up in the sponge tissues to levels that become detrimental to the nudibranchs. No other studies investigating the effects of consuming symbiotic and aposymbiotic sponges on nudibranch predators were found in a review of the literature.

Growth rates of small (≤60 g) and large (>60 g) *Archidoris montereyensis* showed different trends in this study (Fig. 2.7B). Small nudibranchs had increasing growth rates as body size increased while large nudibranchs showed decreasing growth rates. In contrast, Bloom (1981) reported small *A. montereyensis* (≤20 g) had slower growth rates than large (>20 g) *A. montereyensis*. Substantial differences in defining the large category between

studies (60 g vs. 20 g) may account for the different outcomes, as well as the small sample size in the present study. Egg ribbon production rates also differed between the two studies. Daily egg ribbon production observed here was 0.005 g wet weight egg ribbon/g nudibranch, substantially lower than the 0.9 g dry weight egg ribbon/g nudibranch found by Bloom (1981). Bloom's experiments ran almost twice as long, giving the nudibranchs a longer adjustment period in which they may have resumed more normal behaviors. However, Bloom's nudibranchs were maintained in isolation without access to mates, whereas the nudibranchs in this study were maintained in similar-sized pairs and could spend time copulating. Fertilization success in isolated individuals and potential for "selfing" merit study.

Feeding rates of *Archidoris montereyensis* on *Halichondria panicea* were relatively low (0.03 - 0.30 g sponge/g nudibranch • day) and likely underestimated the predation pressure of *A. montereyensis* in natural settings. Similarly low predation rates (0.19 g sponge/g nudibranch • day) have been observed in short-term field caging experiments (Crane 1972) and were reported as underestimates of *A. montereyensis* feeding rates on *H. panicea*. A related nudibranch species, *Archidoris pseudoargus*, had higher feeding rates on *H. panicea* (approximately 0.10 g dry sponge/g nudibranch • day) even though the individuals observed were smaller than *A. montereyensis* in the present study (Carefoot 1967). Carefoot's feeding rate is equivalent to about 0.67 g wet weight sponge/g nudibranch • day, assuming dry weight of *H. panicea* is 15% of wet

weight (Knowlton *unpubl. data*). As both species attain similar adult sizes (approximately 50 g), the higher feeding rate per individual for A. *pseudoargus* could be a result of using small individuals that spend nearly all of their time and energy feeding and growing.

From a community structuring perspective, it appears that the major threat to an established sponge population would be an increase in the predator population rather than high feeding rates of individuals. If collective predation rates exceed sponge growth and repair rates, a net decrease in prey population size should result. Similarly, low predation pressure would have little or no overall effect on prey population size. Individual sponge colonies might disappear, but total population size would not decrease and might even increase. However, it should be noted that nudibranch predation can weaken the attachment of sponge colonies to the substrate, by tunneling underneath the tissue, making the sponge susceptible to removal by wave forces (Knowlton *pers. obs.*). Again, an increased number of predators would be required to overcome the high damage recovery rates of the sponges, but the potential for catastrophic impacts on the sponge population beyond just biomass consumed does exist (see Chapter 3).

Sponge Recovery Rates

Growth rates of several species of sponges have been investigated and most define growth as an increase in ash free dry weight (Barthel 1986, 1989) or

as percentage of initial body mass (Elvin 1976; Frost and Williamson 1980; Osinga et al., 1999; Thomassen and Riisgård 1995; Leichter and Witman 1997). A few studies examined lateral growth of encrusting sponges without reference to the length of the leading edge (Fell and Lewandrowski 1981; Ayling 1983; Contini 1995; Turon et al., 1998). This study looked at the *in situ* colony recovery of substrate area lost due to an artificial predation event rather than growth by volume or weight. Thus, direct comparison of sponge recovery rates with growth rates in other studies is difficult.

For encrusting subtidal sponges (Ayling 1983; Turon et al., 1998) and deep water hexactinellid sponges (Leys and Lauzon 1998), regenerative growth rates into disturbed areas were higher than undisturbed natural growth rates, and that is likely the case for *Halichondria panicea*. While subtidal encrusting species exhibited much faster (22-2900x) repair growth rates relative to natural, undisturbed growth (Ayling 1983), the sponges in this study regenerated at rates ≥3x normal growth. Normal growth rates for sponges in this study were considered to be the slower growth rates observed for grooves that were nearly healed. The high recovery growth rate indicates a response mechanism to tissue damage. The tissue initially regenerating into the disturbed area was generally much thinner than the rest of the colony and thickened over time. These observations concur with Ayling (1983) who performed a similar experiment. It is generally thought that the damaged sponge colonies regenerate a thin tissue layer to quickly recover the substrate area lost due to the disturbance and later

reinforce the region. The unified response to damage is another indication that sponges function as an organism rather than a loose aggregation of cells.

Growth rates ranging from -0.1 to 0.4 cm^2/day for *Halichondria panicea* in this study that approximated normal, undisturbed growth occurred 4 weeks after the artificial predator event. Direct comparisons of growth rates determined in other studies of *H. panicea* are not possible due to differences in methodology for determining growth. However, using conversion factors and certain assumptions, approximate comparisons can be made. For the present study, using an average sponge colony thickness of 3 mm yields an increase in colony volume of up to 0.12 cm^3/day. Converting Barthel's (1986, 1989) ash-free dry-weight growth rates to volume measurements, based on that study's regression analyses, results in an average volume increase of 0.05 cm^3/day. Thomassen and Riisgård (1995) reported specific growth rates of 2.8%/day for *H. panicea* in their study and reported values for Barthel's (1986, 1989) studies of 1.7% and 1.6%, respectively. Growth rates were 1.6 times greater for Thomassen and Riisgård's experiments than those measured by Barthel, indicating that volume increases in sponge colonies were on the order of 0.08 cm^3/day. Both of these growth rate estimates are lower than the maximum measured normal growth rate of *H. panicea* in Alaska, but are within the range of observed rates. Differences observed in growth rates among the three studies may be due in part to the tidal height at which the sponges occurred. The Alaska population was intertidal, experiencing high wave action and tidal currents, while the other studies were on

subtidal sponge populations in areas where water flow was relatively lower. Increased water motion should provide greater nutrient resources to colonies and may decrease the energetic cost of pumping water through their tissues (Vogel 1974, 1988), potentially leaving a larger share of energy resources for growth. Under low current conditions, a sponge colony probably expends relatively more energy moving water through its tissues and less for growth.

Seasonal variations in growth rates for *Halichondria panicea* have been observed in the North and Baltic Seas (Barthel 1986, 1989), as well as for other encrusting sponges (Elvin 1976; Ayling 1983; Turon et al., 1998), with the highest growth rates occurring during the summer. Assuming that there is a similar pattern for sponges in southcentral Alaska, the highest growth rates would occur when *Archidoris montereyensis* predation has a maximal impact on *H. panicea*. The nudibranchs typically recruit in the early spring and would be fairly large by summer, with each individual capable of making feeding grooves similar to the ones artificially inflicted on the sponge colonies. Thus, high summer growth and recovery rates in sponges may contribute to long-term colony stability by reducing the undermining effect of the nudibranchs before winter storms occur.

Summary

Mechanisms structuring a rocky intertidal community dominated by the encrusting sponge *Halichondria panicea* were investigated through three field

and laboratory experiments. In the first experiment, the effect of overlying macroalgae on existing colonies of *H. panicea* was studied using experimental manipulations of permanent quadrats established on the study site. Macroalgae was repeatedly removed from half of the quadrats, while the remaining quadrats were left undisturbed. A year after the final removal, no effect of removing the macroalgae was found on *Halichondria* abundance, suggesting that the presence or absence of overlying macroalgae does not influence sponge abundance. The second experiment was an investigation the predator-prey interaction between *H. panicea* and its primary predator *Archidoris montereyensis*. Nudibranchs consuming symbiotic sponge had higher feeding and egg production rates than individuals eating aposymbiotic sponge. Small nudibranchs (<60 g) had increasing growth rates but large nudibranchs (>60 g) exhibited decreasing growth rates. The final experiment examined *H. panicea*'s response to a simulated predator event. Initial sponge growth rates into experimental feeding grooves were high, indicating a response to tissue damage. Four weeks later, growth rates decreased to approximately normal, undisturbed rates.

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Chapter 3: Convergence in the Time-Space Continuum: A Predator-Prey Interaction[1]

Introduction

Community structure is influenced by many biotic and abiotic factors. Among the former, predation is a key structuring mechanism for some marine communities (Paine 1974). Prey abundance may fluctuate greatly with strength of predator recruitment and persistence except in cases where some of the prey population has a refuge in space or time from predation. Consistent, moderate predation levels on a predictably available prey resource should lead to stable community structure with relatively small fluctuations in predator and prey population densities. Conversely, prey species lacking a refuge from predation are subject to major population fluctuations commensurate with variations in predator abundance.

Community stability can be attained when a prey species is capable of occupying a location (refuge) its predators cannot occupy due to physiological or other constraints. There are a number of intertidal examples. Dense bands of the mussel *Mytilus californicus* occur in the intertidal zone of the U.S. Pacific Northwest, just above the upper foraging limit of the predatory seastar *Pisaster*

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[1]Knowlton, A.L., and R.C. Highsmith. 2000. Convergence in the time-space continuum: a predator-prey interaction. *Marine Ecology Progress Series* 197:285-291.

ochraceous (Paine 1966, 1974). Harpacticoid copepods of the genus *Tigriopus* utilize high level tidepools to escape predators common in lower tidepools (Dethier 1980). The lady crab *Ovalipes ocellatus* is adapted to deep burial in sandy habitats which provides a refuge from a variety of predators, including other crab species (Barshaw and Able 1990). *Choromytilus chorus*, a mytilid mussel, escapes predatory snails by settling on filamentous macroalgae and growing until they are large enough to attain a refuge in size from the predators (Moreno 1995).

Sponges are major components of many benthic communities such as coral reef systems (Hartman 1977), epifaunal assemblages of McMurdo Sound, Antarctica (Dayton et al., 1974), and algal and seagrass communities (Theede 1981, Fell & Lewandrowski 1981). In many cases sponges occupy a significant amount of available substrate, but are not typically spatial dominants. Where sponges do dominate, it is often an assemblage rather than a single species that occupies the space. One example that contradicts both generalities is in McMurdo Sound, Antarctica where 18 species of sponges occupy 55.5% of the available space and 41.8% of the available space is accounted for by a single species (Dayton et al., 1974). The Antarctic sponge community occurs in a physically stable, low-energy environment that appears to favor a poriferan-based community. I investigated the encrusting sponge *Halichondria panicea* in an unpredictable and relatively high wave-energy environment, the rocky intertidal zone in southcentral Alaska.

Halichondria panicea is widely distributed and can be an important component of algal and seagrass communities with relatively small colonies encrusted on blades and shoots (Theede 1981, Fell & Lewandrowski 1981) or on shells and pebbles (Knowlton *pers. obs.*). Ecological and reproductive studies have been carried out on subtidal populations (Ivanova 1981; Barthel 1986, 1988; Witte et al., 1994) but few scientists have investigated *H. panicea* living in intertidal habitats (Palumbi 1984). *H. panicea* is patchily distributed in the rocky intertidal and, in certain locations in Kachemak Bay, Alaska, is the spatial dominant. At one such site *H. panicea* dominated the mid-intertidal for at least 10 years, with low densities of potential molluscan predators such as *Archidoris montereyensis*, *Katherina tunicata*, and *Diadora aspera* present (Highsmith *pers. comm.*). This study was initiated to determine how *H. panicea* maintained dominance at the site by investigating changes in percent cover over time, potential for clone formation, interactions with macroalgae, and predator impacts. I report here on spatial cover over a four year period and impact of high predator (*A. montereyensis*) recruitment in one year.

Materials and Methods

Study Site

The study was conducted at Outside Beach Park, Seldovia, Alaska, near the University of Alaska's Kasitsna Bay Laboratory on the Kenai Peninsula (Fig.

3.1). The region is highly productive due to upwelled water from the Gulf of Alaska entering Kachemak Bay (Sambrotto & Lorenzen 1986). Strong tidal currents resulting from an extreme tidal range of about 8 meters distribute nutrients and food. The study site is a horizontal section of semi-exposed, mid-level rocky intertidal beach approximately 55 m X 10 m, dominated by the encrusting sponge *Halichondria panicea.* The beach is composed of many large boulders and exposed bedrock with the sponge covering the exposed upper surfaces of the rock (Fig. 3.2A). Few colonies were observed growing on the underhanging regions of the rocks. The dorid nudibranch *Archidoris montereyensis* commonly feeds on *H. panicea* at this site (Fig. 3.2B), as do other facultative sponge predators including *Katherina tunicata, Diadora aspera,* and *Henricia* spp. (Knowlton *pers. obs.*).

Observations

Halichondria panicea abundance was measured from August 1994 through June 1999 in ten permanent 0.25 m^2 quadrats (Fig. 3.2C, D). Areal coverage was estimated monthly April-August and bimonthly September-March using a quadrat frame with a string grid having 81 intersections. Point counts were recorded for each intersection to estimate species composition of primary space occupiers below the canopy. An additional ten and five 0.25 m^2 quadrats in August and September 1997, respectively, were thrown at random to supplement the permanent quadrat coverage estimates. Data were taken in four

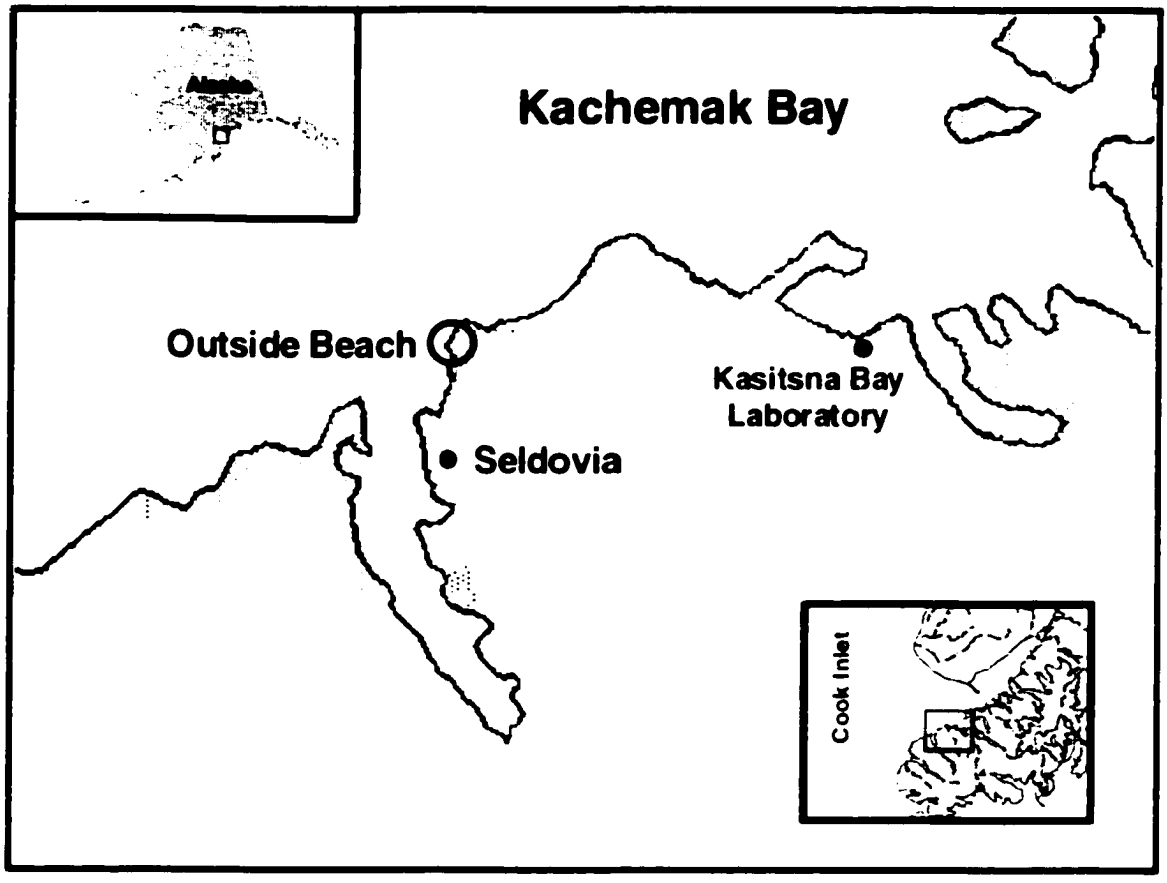
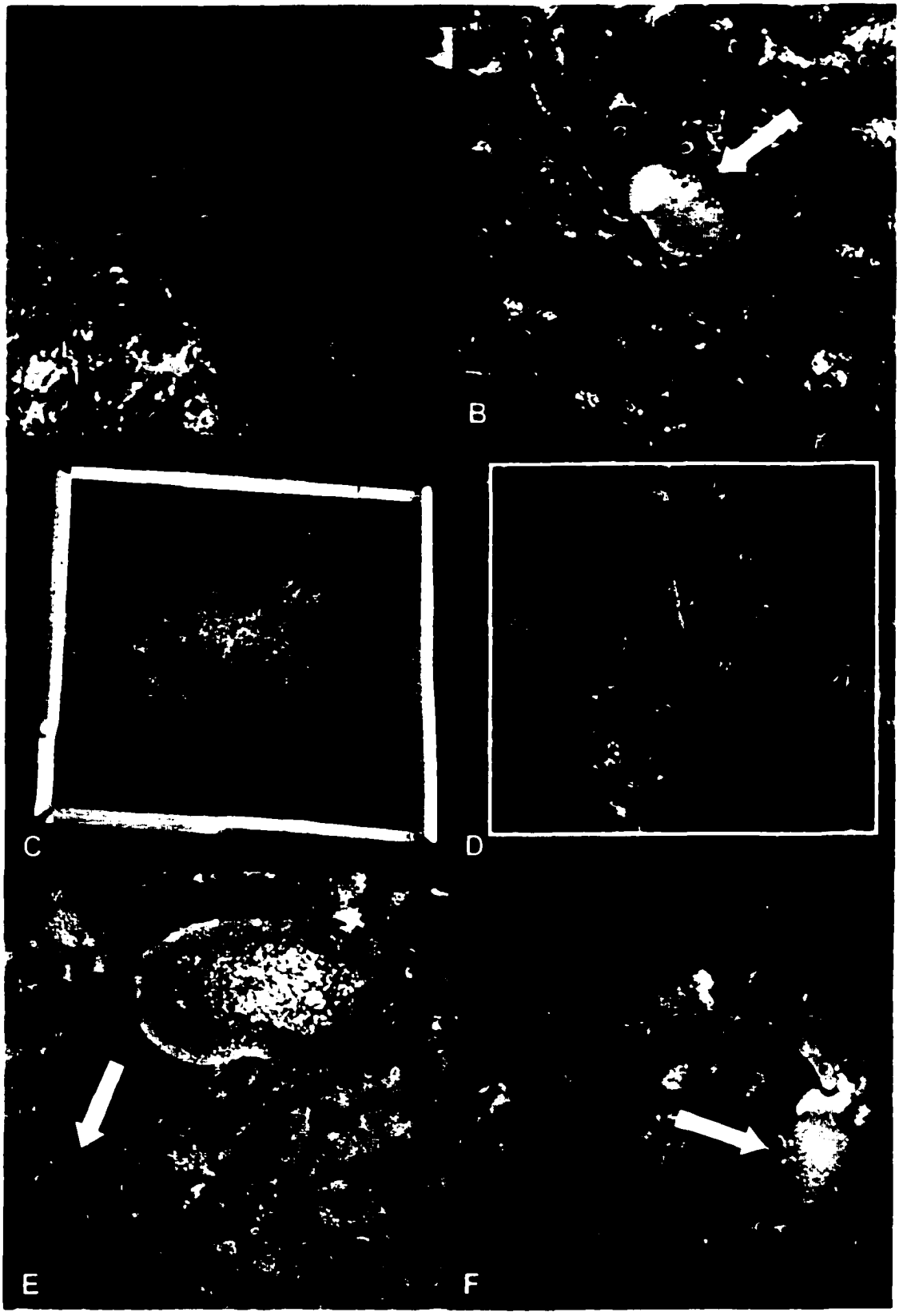


Figure 3.1: Location of the study site at Outside Beach Park near Seldovia, Alaska. Insets: regional context.

Figure 3.2: Study site photograhs. (A) View of the study site in 1994 with numerous large colonies of *Halichondria panicea* evident. (B) The nudibranch *Archidoris montereyensis* (~25 mm long; arrow) burrowed into the sponge *H. panicea*. (C) One of ten 0.25 m^2 permanent quadrats in 1994 showing *H. panicea* dominance. (D) The same quadrat as in (C) in 1998 showing complete absence of sponge. Quadrat outline has been added for size reference. (E) A fresh feeding groove (arrow) made by *A. montereyensis* (~70 mm long). (F) A tunnel (indicated by tool) created by a small nudibranch (~20 mm; arrow).



categories, *H. panicea*, macroalgae, open rock, and other, and histograms of percent cover were plotted. Only the *H. panicea* data will be presented here. A photographic record of the permanent quadrats and of the site in general was compiled for most sampling dates.

The permanent quadrats were originally established to investigate the influence of algal canopy on *Halichondria panicea* abundance. Half of the quadrats were randomly chosen for removal of overlying macroalgae, while the remainder were untouched. Algal removal occurred in July 1994 and monthly April-July 1995. After July 1995 macroalgae were no longer removed and all quadrat flora were allowed to grow without experimental intervention. Percent cover data collected from August 1994-August 1996 were analyzed for treatment effects. A t-test of the slope of regression lines showed no significant difference ($\alpha = 0.05$) in *H. panicea* percent cover between treated and control quadrats so data were pooled for the entire study period for analysis of predator impacts.

Archidoris montereyensis were counted and measured at the study site each July from 1994-1997, with additional counts in May, August, and September 1997, January through June 1998, and June 1999. Nudibranch body lengths were estimated by holding a plastic rule just above the animals without disturbing them. This method does not yield precise lengths, but is adequate for comparison of relative nudibranch sizes over time at a site.

Results

Halichondria panicea is not a cryptic species at the study site, but rather, occupies upper surfaces of boulders (Fig. 3.2A) usually colonized by barnacles, mussels, or algae. Average *H. panicea* cover was 53.4% ± 9.9% through fall of 1996 (Fig. 3.3). Visits to the site in early spring of 1997 revealed that the sponge colonies overwintered with few indications of major mortality events, although percent cover data were not collected at that time as the original cover study had concluded. During subsequent site visits in 1997, visual estimates of *Halichondria panicea* percent cover declined from 40% in May to 15% in July. By August 1997, when 10 permanent quadrats and 10 haphazardly placed quadrats were censused, only rock surfaces and algae were found (Fig. 3.2D). Just a single sponge colony was found on the entire study site. Through June 1999, no *H. panicea* were located within the permanent quadrats. Three small colonies were located on site amongst the holdfasts of the ribbon kelp *Alaria marginata* during the summer of 1998, but have subsequently disappeared.

The nudibranch *Archidoris montereyensis* is a specialized predator on *Halichondria panicea* (Bloom 1976) and is generally found aggregated on or near its sponge prey. I have searched many beaches within a few kilometers of the study site for other sponge-dominated locations and the presence of *A. montereyensis*. Occasionally a lone nudibranch will be found that is not near *H. panicea*, but most are found in pairs feeding on the sponge or producing

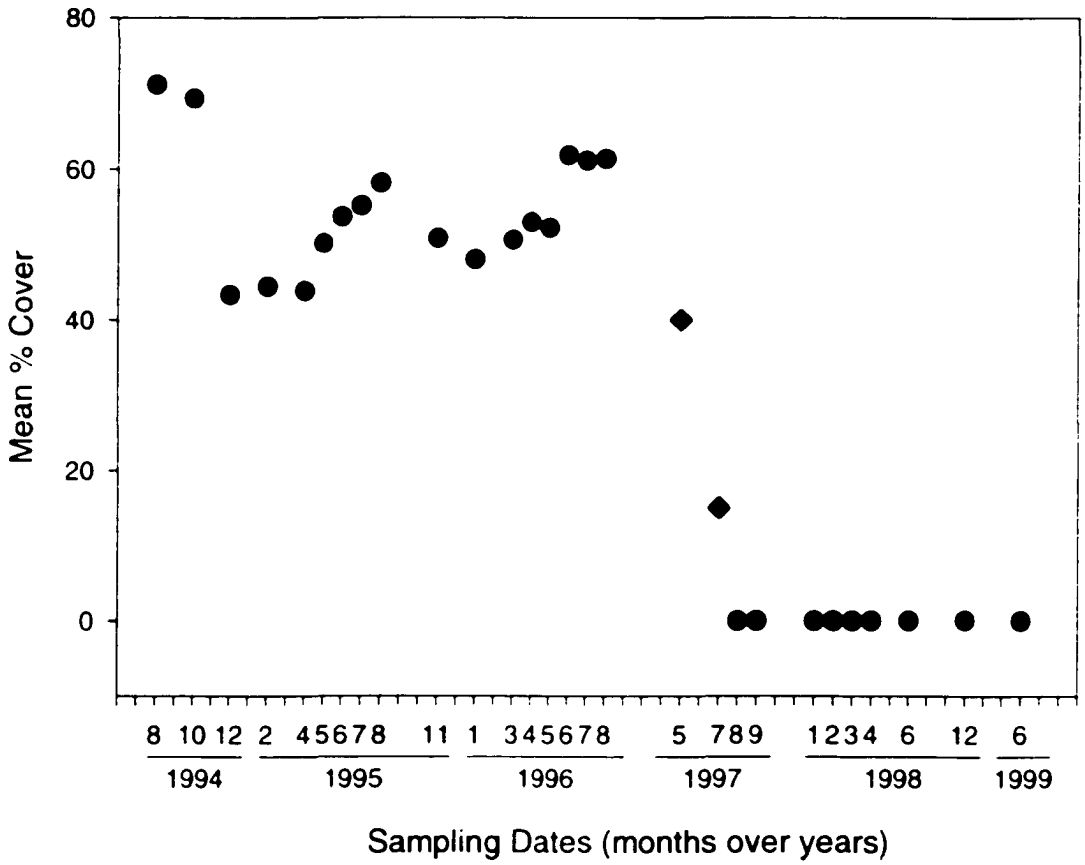


Figure 3.3: *Halichondria panicea* mean percent cover at ten permanent quadrats from August 1994 through June 1999. Points for May and July 1997 are based on visual estimates of sponge percent cover (◆). Data for 10 additional random quadrats in August 1997 and 5 in September 1997 are included. Numbers below x-axis denote sample month and year. Spaces between points represent months with no observations; width is not uniform.

gelatinous egg ribbons adjacent to sponges. Adult *A. montereyensis* are relatively easy to observe because of their yellow color, presence of egg ribbons, and grazed-out feeding tracks in the sponge colony (Fig. 3.2E). In thicker portions of colonies, the nudibranch feeding grooves may turn into tunnels (Fig. 3.2F) that presumably result in undermining the attachment of the colony to the rock surface. As nudibranchs creep on a large foot also adapted for attachment, they are not very mobile and are unlikely to migrate any appreciable distance, especially between boulders separated by unstable gravel (Fig. 3.2A). Therefore, the aggregation of *A. montereyensis* individuals with *H. panicea* is likely due to selective settlement of the nudibranch larvae in response to a chemical cue produced by or associated with the sponge, which they feed upon preferentially (Bloom 1976).

Although it has been stated that *Archidoris montereyensis* larvae settle within two hours of hatching (Morris et al., 1980), I agree with Strathmann (1987) that such rapid settlement is unlikely. The mean *A. montereyensis* egg diameter given in Strathmann (1987) is 81.5 μm. For eggs preserved 24 hours after deposition, I found a mean diameter of 88.5 ± 13.9 μm (n = 25, range 75-125 μm). Such small eggs suggest planktotrophic development rather than direct development with immediate post-hatching settlement. This view is further supported by Hurst's (1967) data. *A. montereyensis* larvae probably feed and disperse over a period of several days to a few weeks.

Total numbers of the nudibranch *Archidoris montereyensis* present at the site ranged from 12 to 42 from 1994-1996 (Table 3.1, Fig. 3.4A). Strong recruitment in 1997 resulted in an average population of 156 *A. montereyensis* on site from May to July (Fig. 3.4B). Because of the low numbers of individuals on site in 1996, high winter survival is not a likely explanation of the increase in nudibranch numbers in 1997. A regression of monthly mean nudibranch length for 1997 suggests a recruitment event occurred early in 1997 (Fig. 3.4C). After July, the abundance of nudibranchs declined to 32 individuals, commensurate with sponge reduction (Fig. 3.4B). By September, only one small sponge colony and 7 nudibranchs were present at the site. From January through June 1998, very few nudibranchs were observed and were not in the vicinity of the few remaining *Halichondria panicea* colonies. These individuals were possibly feeding upon alternative prey or small, cryptic colonies of *H. panicea* that we did not notice. Since 1998 through July 1999, no nudibranchs have been seen at the study site.

The study site is on a semi-exposed rocky intertidal shore (Fig. 3.2A), more exposed than where one would expect to find tens to hundreds of nudibranchs aggregated. I postulate that the sponge colonies being preyed upon provide shelter for the nudibranchs which occur around the edges of colonies and in grooves and tunnels grazed in the sponges (Fig. 3.2B, E, F), all of which reduce drag forces. When the sponges are removed, the nudibranchs are probably much more susceptible to removal by wave action. Also, the

Table 3.1: *Archidoris montereyensis* abundance and size-frequency distribution, 1994 to 1999. Nudibranch lengths were estimated to the nearest millimeter.

Date	Number by Size Category (mm)								
	total	<10	10-19	20-29	30-39	40-49	50-59	60-69	70+
Jul 1994	27	0	0	1	6	7	9	2	2
Jul 1995	42	0	0	3	12	10	9	5	3
Jul 1996	12	0	1	1	3	2	4	0	1
May 1997	177	0	6	30	52	64	20	4	1
Jul 1997	135	0	0	2	17	24	28	34	30
Aug 1997	32	0	0	0	6	9	10	5	2
Sep 1997	7	0	0	0	1	6	0	0	0
Nov 1997	2	0	0	1	0	1	0	0	0
Jan 1998	0	0	0	0	0	0	0	0	0
Feb 1998	2	1	0	1	0	0	0	0	0
Mar 1998	1	0	0	1	0	0	0	0	0
Apr 1998	1	0	0	0	1	0	0	0	0
Jun 1998	0	0	0	0	0	0	0	0	0
Jun 1999	0	0	0	0	0	0	0	0	0

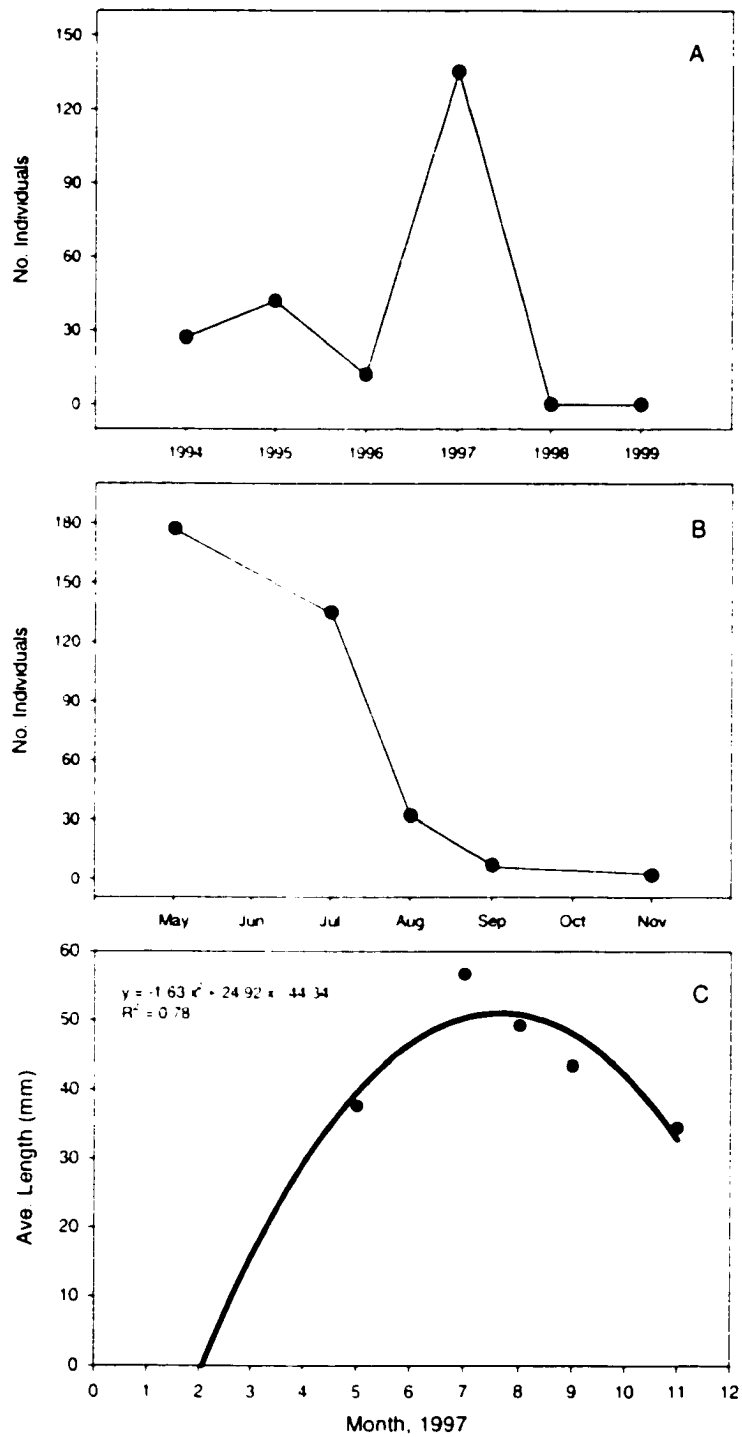


Figure 3.4: *Archidoris montereyensis* population characteristics. (A) Annual census 1994 to 1999 taken each July (1998 and 1999 censuses taken in June). (B) May to November 1997 counts. (C) Regression of nudibranch mean lengths over time in 1997. Numbers below x-axis denote calendar month.

sponge colonies are probably more susceptible to wave removal due to grazing impacts and resident nudibranchs may be carried away with them.

Discussion

The intertidal sponge *Halichondria panicea* apparently relies on a patchy, unpredictable distribution, both spatially and temporally, as a refuge from its major predator. This system is similar to the predator-prey relationship found for the sea pen *Ptilosarcus gurneyi* and its main predator *Mediaster aequalis* (Birkeland 1974) in which both species utilize planktonic larvae for dispersal. While community dynamics and compositions differ between the rocky intertidal inhabited by *H. panicea* and the sandy subtidal habitats occupied by *P. gurneyi*, the mechanisms that appear to structure the communities are the same. A key prey species, also termed "key-industry species" (Elton 1947), can shape an entire community, determining species composition, abundance and stability over time. At the study site in Kachemak Bay, Alaska, *H. panicea* was the key-industry species for over a decade. Even though it is abundant in the region and potential recruits should be numerous, as of June 1999, the site once dominated by *H. panicea* is open rock with heavy recruitment of annual macroalgae occurring. The community is undergoing a restructuring brought about by the removal of the dominant space occupier by its primary predator.

Over evolutionary time, predators must adapt to changes in the defense mechanisms of their preferred prey, remain a generalist, or go extinct. Many studies have focused on predators modifying behaviors or life-history strategies as their prey species develop new chemical and morphological defense mechanisms or find new refugia (Birkeland et al., 1971; Bloom 1976; Vermeij 1987). In cases where prey are patchily distributed in habitats in which predators are not physiologically or otherwise excluded, and the major predator has limited adult mobility, then the patchy, unpredictable location of the prey may serve as a refuge. The random distribution of prey populations within a habitat is a result of larval dispersal by currents. In some years and locations, recruitment may be high due to favorable planktonic conditions, current patterns, and availability of a suitable site, perhaps due to a recent disturbance that removed resident species. Predators with larval dispersal have the same obstacles to and potentials for successful recruitment in suitable habitats with the added constraint of locating within-habitat prey patches. High predator recruitment may occur when all requisite conditions are met and the larvae detect cues indicative of prey presence (Mauzey et al., 1968; Birkeland et al., 1971; Birkeland 1974). In this case, a refuge site is lost as both prey and predator eventually become locally extinct at the site. This type of system may provide long-term, large-scale stability to a species but only short-term stability to a population at a given location. The predator-prey relationship of *Archidoris montereyensis* and *Halichondria panicea* is an example of a chase through space and time with

convergence resulting in extreme population fluctuations and an unstable community.

Summary

Predation is a key structuring mechanism for some marine communities. Prey abundance may fluctuate with strength of predator recruitment and persistence except in cases where some of the prey population has a refuge in space or time from predation. The sponge *Halichondria panicea* is patchily distributed in the rocky intertidal on the south shore of Kachemak Bay, southcentral Alaska, and in certain locations is the spatial dominant. This long-lived sponge is dispersed by planktonic larvae. At one site *H. panicea* has dominated the mid-intertidal for at least 10 years. Percent cover estimates show that *H. panicea* averaged 53.4% ± 9.9% cover from August 1994 through August 1996. A major predator on *H. panicea* is the nudibranch *Archidoris montereyensis*, which is also planktonically dispersed and has an annual life cycle. Predators with larval dispersal have the same obstacles to and potential for recruitment in suitable habitats as planktonically dispersed prey with the added constraint of locating within-habitat prey patches. Total numbers of *A. montereyensis* at the study site (550m^2) ranged from 12 to 42 from 1994-1996. In the spring of 1997, strong recruitment resulted in an average population of 156 *A. montereyensis* on site from May to July. Percent cover of *H. panicea* declined

from 40% in May to 15% in July. By August 1997, sponge was absent at the study site and the number of nudibranchs declined to 7 individuals by September. Even though *H. panicea* is abundant in the region and potential recruits should be numerous, as of June 1999, the site once dominated by *H. panicea* is open rock with heavy recruitment of annual macroalgae occurring. The predator-prey relationship of *A. montereyensis* and *H. panicea* is an example of a chase through space and time with convergence resulting in extreme population fluctuations and an unstable community.

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Chapter 4: Seasonality of Egg Production of Intertidal Sponges in Southcentral Alaska

Introduction

The reproductive cycles of many subtidal marine sponge populations have been investigated (Fell and Jacob 1979; Ayling 1980; Barthel 1986; Wapstra and van Soest 1987; Tanaka-Ichihara and Wantanabe 1990; Kaye and Reiswig 1991; Witte and Barthel 1994; Corriero et al., 1996), but only a few studies have focused on sponge reproduction in intertidal habitats (Elvin 1976; Ivanova 1981; Vethaak et al., 1982; Yereskovskiy 1995). Inherent in an intertidal existence are the added stresses of dealing with an increased number and range of abiotic factors including desiccation, temperature, exposure to air, and UV radiation. Thus, the timing and allocation of resources for reproduction may vary between subtidal and intertidal populations of the same or similar species. Habitat features such as wave-exposure, sedimentation rate, substrate type or shading might also affect the occurrence and extent of reproductive events (Vethaak et al., 1982). For example, sponge colonies might have to allocate a larger share of resources for producing spicules and tougher exterior surfaces to withstand high wave action, resulting in fewer resources for reproduction.

Environmental signals at high latitudes are typically more intense and more sharply defined than at lower latitudes. Day lengths and air temperatures

vary dramatically between summer and winter months, influencing other environmental signals such as water temperature and stratification, ice formation, and freshwater runoff (Burbank 1977).

Exposure to various combinations of environmental signals and stresses should produce behavioral or physiological adaptive responses. For sponges, there are several known methods of propagation. Production of gametes is common and asexual reproductive modes include gemmule formation, budding, and fragmentation (Ayling 1980; Berquist et al., 1970; Fell 1974; Simpson 1980; Jokiel et al., 1982). Budding and fragmentation may require less energy than reproducing sexually, but are more opportunistic and may rely on mechanical stress to carry out the process. Investigating the relative importance of sexual and asexual reproductive modes in a sponge population will give insight into energy budgets and constraints vs. environmental conditions.

At the initiation of this study, all sponge populations under investigation were thought to be the same species, *Halichondria panicea*, based on local knowledge and identifications. Recent genetic work (Chapter 5, 6) revealed the presence of other species within the populations. Identifications for the purpose of this study are based on the most probable matches with available ecological and morphological data.

The objectives of this study were to determine the seasonal cycles of oocyte production of four intertidal sponge populations of *Halichondria panicea*, two in sheltered locations and two in exposed locations, in southcentral Alaska

and to compare them to published results for the same species in other geographic locations. Due to the strong seasonal light and temperature signals in southcentral Alaska, distinct seasonal periodicity in sponge reproductive activity was predicted. In addition, peak in reproductive activities for sponge colonies at semi-exposed, hard substrate habitats should be at a different time than for colonies in protected, soft-sediment habitats. Any differences found were expected to reflect adaptations to nutrient-rich, wave-washed conditions at semi-exposed sites vs. adaptations to warmer, sedimentary conditions at sheltered sites. Further, a preliminary evaluation of the relative importance of sexual vs. asexual reproduction for sponges in southcentral Alaska will be presented.

Materials and Methods

Study Sites

Four intertidal study sites were established on the southern shore of Kachemak Bay, which is located on the Kenai Peninsula in southcentral Alaska (Fig. 4.1). Two study locations, Outside Beach and Camel Rock, are semi-exposed rocky substrate habitats and the other two, Jakolof Bay and Seldovia Bay, are protected soft-sediment sites. The region is highly productive due to upwelled waters from the Gulf of Alaska entering Kachemak Bay (Burbank 1977, Sambrotto and Lorenzen 1986). Strong tidal currents resulting from an extreme

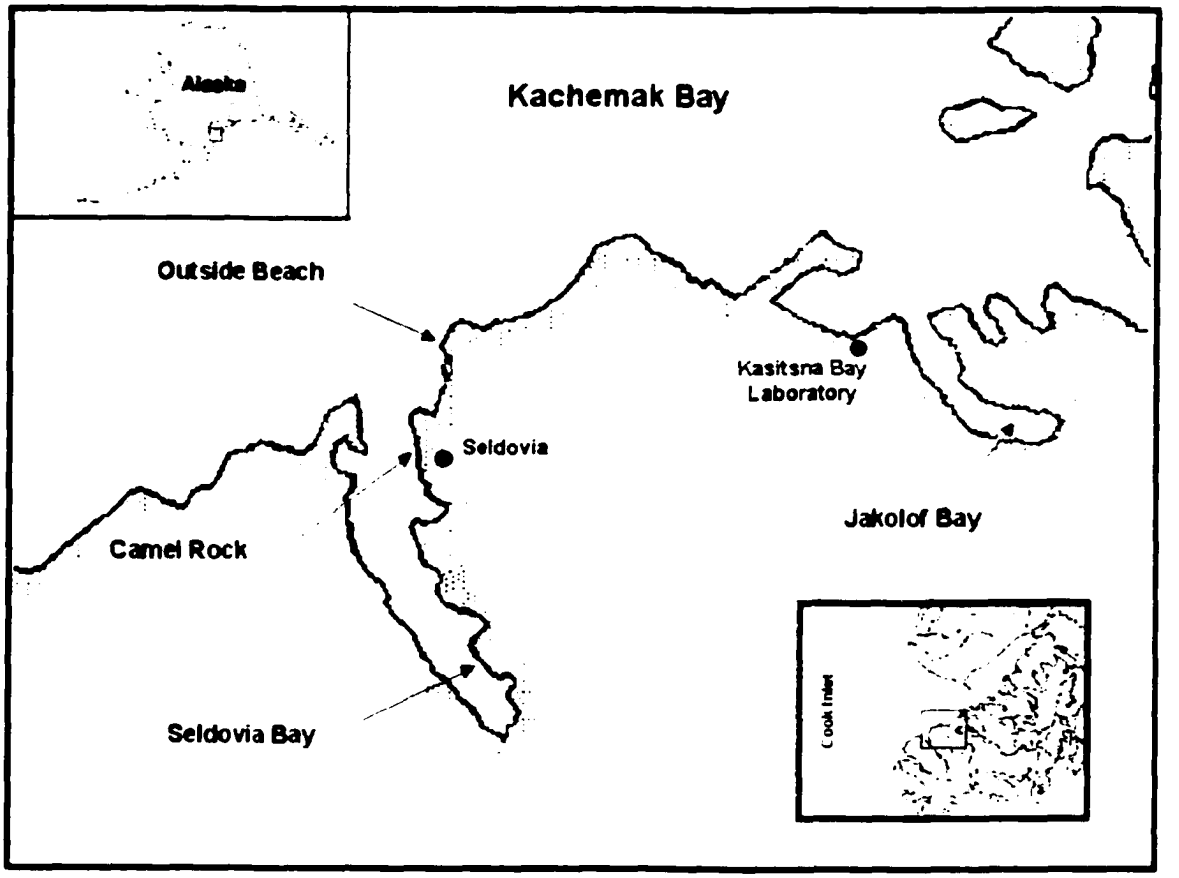

Figure 4.1: Map showing the locations of the four study sites in Kachemak Bay, Alaska. Insets: regional context.

tidal range of about 8 meters distribute nutrients and food. The sheltered sites are seldom, if ever, impacted by waves whereas the semi-exposed sites are routinely wave-washed and occasionally experience storm waves a few meters in height, especially in fall and winter (Knowlton *pers. obs.*)

Sites in similar habitat types were comparable in several physical and environmental parameters. The sponge populations occurred at similar tidal heights within habitats. For semi-exposed sites the average tidal height for the study populations was 0.0 meters and +0.3 meters mean low low water (MLLW) at Outside Beach and Camel Rock, respectively. For sheltered sites, the Jakolof Bay population was at +1.5 meters and the Seldovia Bay population at +0.9 meters MLLW. Both soft-sediment sites were located near the head of a bay with a freshwater source. Salinity at sheltered sites ranged from 27-30‰ at the surface. Water was well mixed at the hard-substrate sites resulting in a constant salinity of 30-31‰. Water temperatures at all sites varied 2-3°C between the surface layer and 10 meters depth. Seasonal changes in subsurface water temperature in nearby Kasitsna Bay were monitored from 1992-1995 using a subsurface temperature recorder (Fig. 4.2A). A relative measure of water motion (wave action, tidal exchange, current) at each site was obtained by measuring dissolution rates of gypsum cylinders (Petticrew and Kalff 1991; Highsmith et al., 1995). Average dissolution rates of four cylinders per site were obtained monthly for nearly a year by dividing total weight loss by total submergence time. Similar habitat sites showed similar seasonal patterns of integrated hydrodynamics

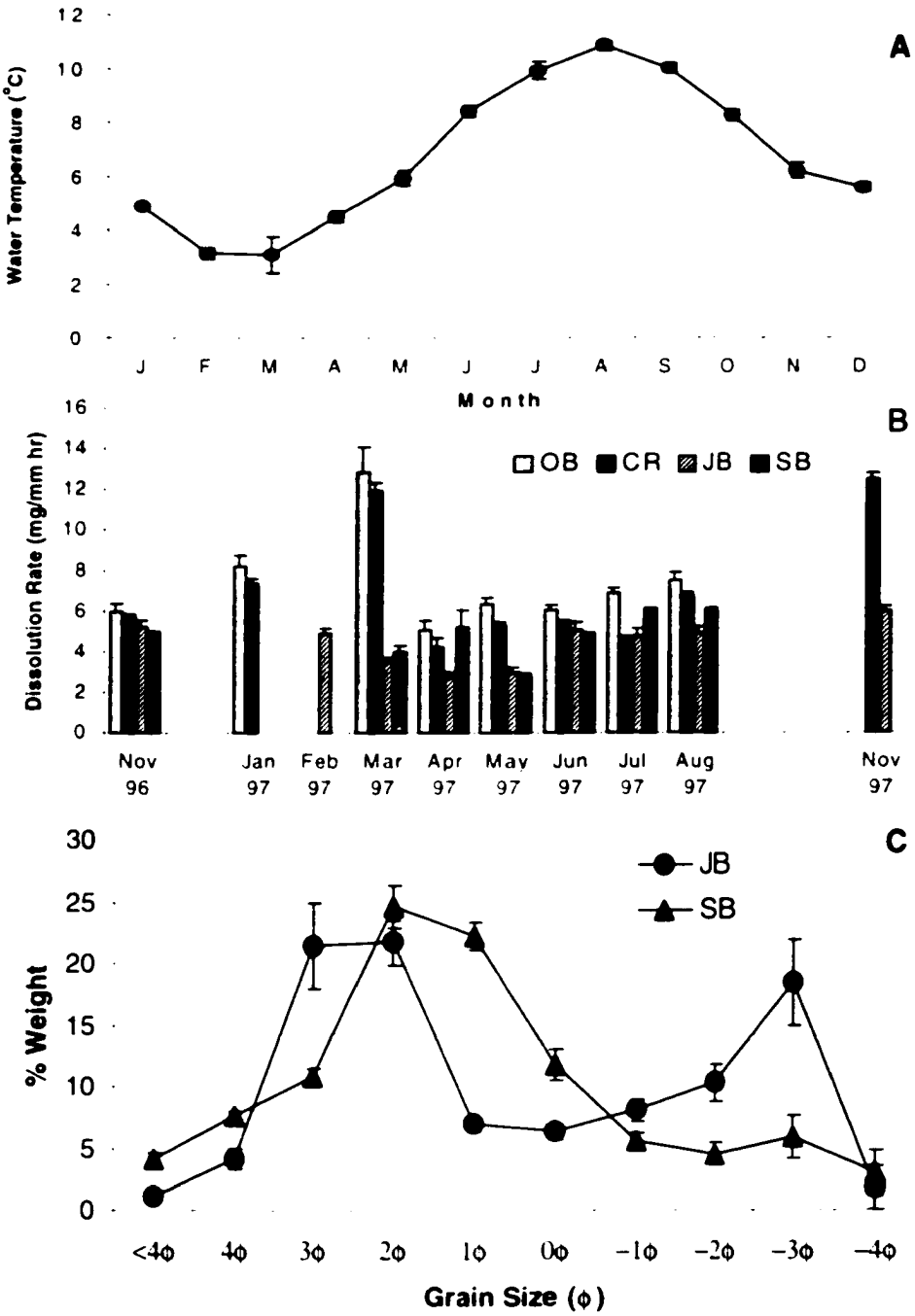

Figure 4.2: Physical characteristics of the study sites. A. Monthly water temperature for Kasitsna Bay, Alaska, 1992-1995. Mean ± 1 standard error (SE). B. Dissolution rate of gypsum cylinders set out for 3-4 days at each study site and standardized for actual submergence time. OB = Outside Beach, CR = Camel Rock, JB = Jakolof Bay, SB = Seldovia Bay. Vertical bar = 1 SE. C. Grain size analysis of 10 sediment cores per site collected from Jakolof Bay (JB) and Seldovia Bay (SB). Mean ± 1 SE.

(water motion) (Fig. 4.2B). Sediment grain size was also consistent within habitats. Both hard substrate sites were composed of exposed bedrock and large boulders. Grain size analysis of 10 sediment cores from each protected site showed similar trends in sediment size composition, but two-sample t-tests of size classes revealed significant differences in eight out of ten size categories ($\alpha = 0.05$) (Fig. 4.2C).

Numerous sponge colonies were present at all sites and populations had persisted at each site for several years prior to this investigation (Highsmith, *pers. com.*, Knowlton *pers. obs.*). The rocky habitat populations consisted of the cosmopolitan species *Halichondria panicea*, while those at soft substrate sites were subsequently found to be a complex of *Halichondria bowerbanki* and an unidentified *Halichondria*-like species (Chapter 6). For purposes of this chapter, *H. bowerbanki* will be used for the soft-substrate sponge populations. Further investigation and identification of the species studied are discussed in Chapter 6.

Sample Collection and Processing

Monthly tissue samples from the first ten colonies larger than 100 cm^2 at each site were collected from October 1996 through September 1997. Samples were preserved in formalin and transported to the laboratory in Fairbanks for histological examination. A portion of tissue approximately 3 mm x 3 mm x 3 mm was excised from each sample and placed in a 5% hydrofluoric acid wash to dissolve the siliceous spicules. The subsamples were then processed through

an alcohol dehydration series and embedded in paraffin for sectioning. Serial cross-sections 8 μm thick were mounted and stained using Papanicolaou OG-6, hematoxylin, and eosin.

Oocyte Diameters and Developmental Stages

Each sample was viewed using a compound light microscope (Leitz Laborlux S) with a video connection to a desktop computer (Dell Dimension XPS) to quantify oocyte presence and developmental stage. Oocyte diameters were measured using a digitizing tablet and image analysis software (SigmaScanPro). Feret diameter, which is the diameter of a hypothetical circle having the same area as the object being measured, was utilized in order to control for the irregular shape of most oocytes. Only eggs with both a nucleus and nucleolus present were measured to prevent an egg from being counted more than once and to attempt to standardize the cross-section of the egg being measured. Up to the first 100 oocytes encountered in a sample were counted and measured. Oocytes were allocated to developmental categories based primarily on feret diameter, but also on morphological characteristics such as placement of nucleus and presence of yolk granules (Witte and Barthel 1994): stage I oocytes ≤20 μm, stage II oocytes 20-50 μm, stage III oocytes 50-90 μm, stage IV oocytes 90-120 μm. Embryos were larger than 120 μm and lacked a single large nucleus.

An estimate of oocyte density was obtained by dividing the number of oocytes counted by the volume of sample viewed. The area analyzed was

estimated by measuring the cross-sectional area of each tissue slice viewed with the digital analysis software. Multiplying the total area by 8 μm, the slice thickness, gave the estimated volume of sponge tissue analyzed.

Statistical Analyses

Logistic regression analysis of the data was performed based on presence or absence of a particular oocyte developmental stage within the sampled colonies. The data were categorized by quarter, season, site and habitat (Table 4.1B). Comparisons were considered statistically significant at the $\alpha = 0.05$ level.

Results

Logistic regression analyses of the occurrence of oocyte developmental stages within the sample populations did not reveal broad patterns, but indicated localized or developmental stage-specific trends (Table 4.1A). No single treatment effect was significant for every developmental stage tested, but every developmental stage had at least one significant treatment effect. Pairwise comparisons within treatment effects having more than two options (i.e. quarter, site) were performed (Table 4.2, 4.3) to explain causality of significant effects.

Table 4.1: Logistic regression analyses of the occurrence of sponge oocyte developmental stages. A. Overall results of treatment effects. Asterisk (*) = significant effect at $\alpha = 0.05$, double asterisk (**) = significant at $\alpha = 0.01$, ns = not significant. B. Definition of treatment effects.

A

	Oocyte Stage				
	Stage I	**Stage II**	**Stage III**	**Stage IV**	**Embryos**
Season	ns	ns	**	ns	**
Quarter	ns	*	**	ns	ns
Habitat	ns	ns	ns	ns	**
Site	**	ns	ns	*	*

B

Season
Winter (October 1996-March 1997)
Summer (April-September 1997)

Quarter
October-December 1996
January-March 1997
April-June 1997
July-September 1997

Habitat
Semi-exposed, rocky
Protected, soft-sediment

Site
Outside Beach
Camel Rock
Jakolof Bay
Seldovia Bay

Table 4.2: Pairwise comparisons within quarter treatment effects for each oocyte developmental stage based on logistical regression analyses. > = direction of greater probability of oocyte developmental stage occurrence, ns = no significant difference.

	Oct-Dec 1996	**Jan-Mar 1997**	**Apr-Jun 1997**	**Jul-Sep 1997**
Oct-Dec 1996				
Stage I				
Stage II				
Stage III				
Stage IV				
Embryos				
Jan-Mar 1997				
Stage I	ns			
Stage II	Oct>Jan			
Stage III	Oct>Jan			
Stage IV	ns			
Embryos	ns			
Apr-Jun 1997				
Stage I	ns	Apr>Jan		
Stage II	ns	Apr>Jan		
Stage III	Oct>Apr	ns		
Stage IV	ns	ns		
Embryos	ns	Jan>Apr		
Jul-Sep 1997				
Stage I	ns	ns	ns	
Stage II	ns	Jul>Jan	ns	
Stage III	Oct>>Jul	Jan>>Jul	Apr>>Jul	
Stage IV	Oct>>Jul	Jan>>Jul	Apr>>Jul	
Embryos	Oct>>Jul	Jan>>Jul	Apr>>Jul	

Table 4.3: Pairwise comparisons within site treatment effects for each oocyte developmental stage based on logistical regression analyses. > = direction of greater probability of oocyte developmental stage occurrence, ns = no significant difference.

	Outside Beach	Camel Rock	Jakolof Bay	Seldovia Bay
Outside Beach				
Stage I				
Stage II				
Stage III				
Stage IV				
Embryos				
Camel Rock				
Stage I	ns			
Stage II	ns			
Stage III	ns			
Stage IV	ns			
Embryos	ns			
Jakolof Bay				
Stage I	OB>JB	CR>JB		
Stage II	ns	ns		
Stage III	ns	ns		
Stage IV	ns	JB>CR		
Embryos	ns	CR>JB		
Seldovia Bay				
Stage I	ns	ns	SB>JB	
Stage II	ns	ns	SB>JB	
Stage III	ns	ns	ns	
Stage IV	ns	ns	JB>>SB	
Embryos	OB>>SB	CR>>SB	JB>>SB	

For seasons, winter vs. summer was significant for stage III oocytes and embryos. In both cases, stage III oocytes and embryos are six times more likely to be found during the winter than during the summer. Having later stages of oocyte development be more prevalent during a specific time period supports the hypothesis that there is a strong seasonal signal in reproductive activities for sponges in southcentral Alaska.

While quarter showed an overall statistically significant effect for stage II and stage III oocytes, a more interesting trend was elucidated by the pairwise comparisons (Table 4.2). Large oocytes (stage III and IV) and embryos were much more likely to occur during October 1996-June 1997, the first three quarters of the sampling period, than during the last three months of sampling, July-September 1997. This suggests a post-reproductive period of lower activity occurring after large oocytes had matured, been fertilized, and developing embryos had been released as larvae, and supports the seasonality hypothesis.

Habitat, semi-exposed rocky vs. protected soft-sediment, was only significant for embryos. The probability of finding an embryo in a sponge colony from a rocky exposed habitat is nine times greater than the probability of finding one from a soft-sediment colony. This indicates either a shift in the relative importance of sexual vs. asexual reproduction between colonies in different habitats, a difference in the reproductive effort (number of eggs and embryos) produced, or genetic (or species) divergence.

The overall effect of sampling site was significant for stage I and stage IV oocytes, as well as for embryos. In pairwise comparisons, two trends stand out (Table 4.3). First, Outside Beach, Camel Rock, and Seldovia Bay were three to five times more likely to have stage I oocytes present than at Jakolof Bay. Secondly, embryos rarely occurred at Seldovia Bay compared to other sites, which is illustrated in the lack of embryos found at Seldovia Bay.

Seasonality of Reproduction

Sponge populations in Kachemak Bay exhibited a seasonal signal in reproductive activity. Oocyte growth and yolk accumulation started in mid- to late-summer for populations at exposed sites (*Halichondria panicea*) and mid-winter for protected sites (*H. bowerbanki*) (Fig. 4.3, 4.4). Large oocytes (stages III and IV) and embryos were present in *Halichondria panicea* samples from the rocky exposed sites during winter months (Fig. 4.4A, B). In *H. bowerbanki* populations at sheltered sites, large oocytes and embryos tended to occur in the spring (Fig. 4.4C, D). At all sites, low levels of small oocytes (stages I and II) were present throughout the sampling period indicating a degree of reproductive readiness at all times. A trend towards larger oocytes and embryos over time is apparent for all sites except Seldovia Bay and reflects the growth of the current oocyte cohort (Fig. 4.4). The reduced occurrence of advanced oocyte stages is problematic. The low degree of synchronicity within the populations could contribute to the failure to detect of large oocytes, which

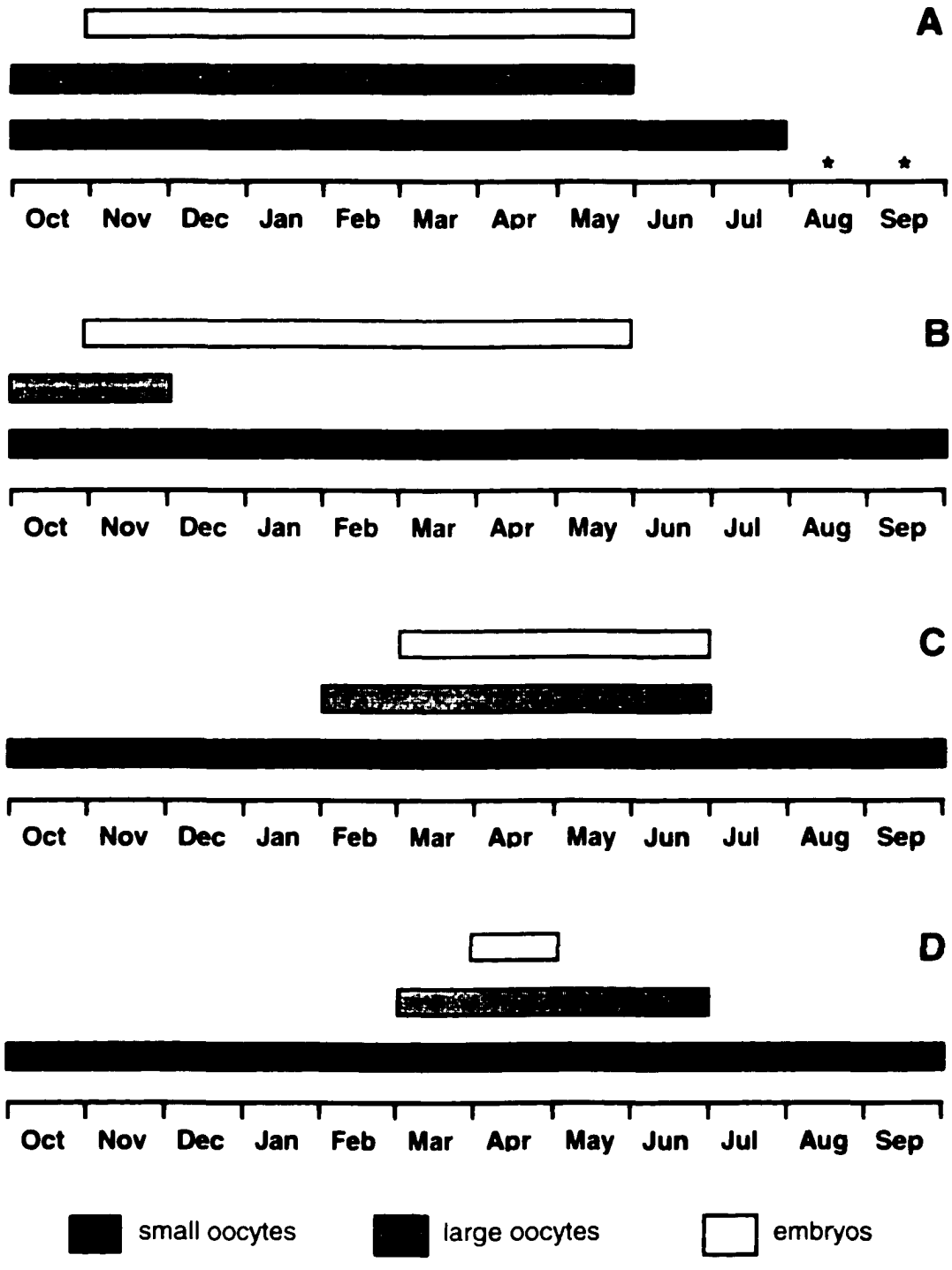

Figure 4.3: Seasonal patterns of female reproductive activities, October 1996-September 1997. Small oocytes include stage I and stage II oocytes. Large oocytes are stage III and stage IV. A. Outside Beach, B. Camel Rock, C. Jakolof Bay. D. Seldovia Bay. Asterisk (*) = no data, no samples collected.

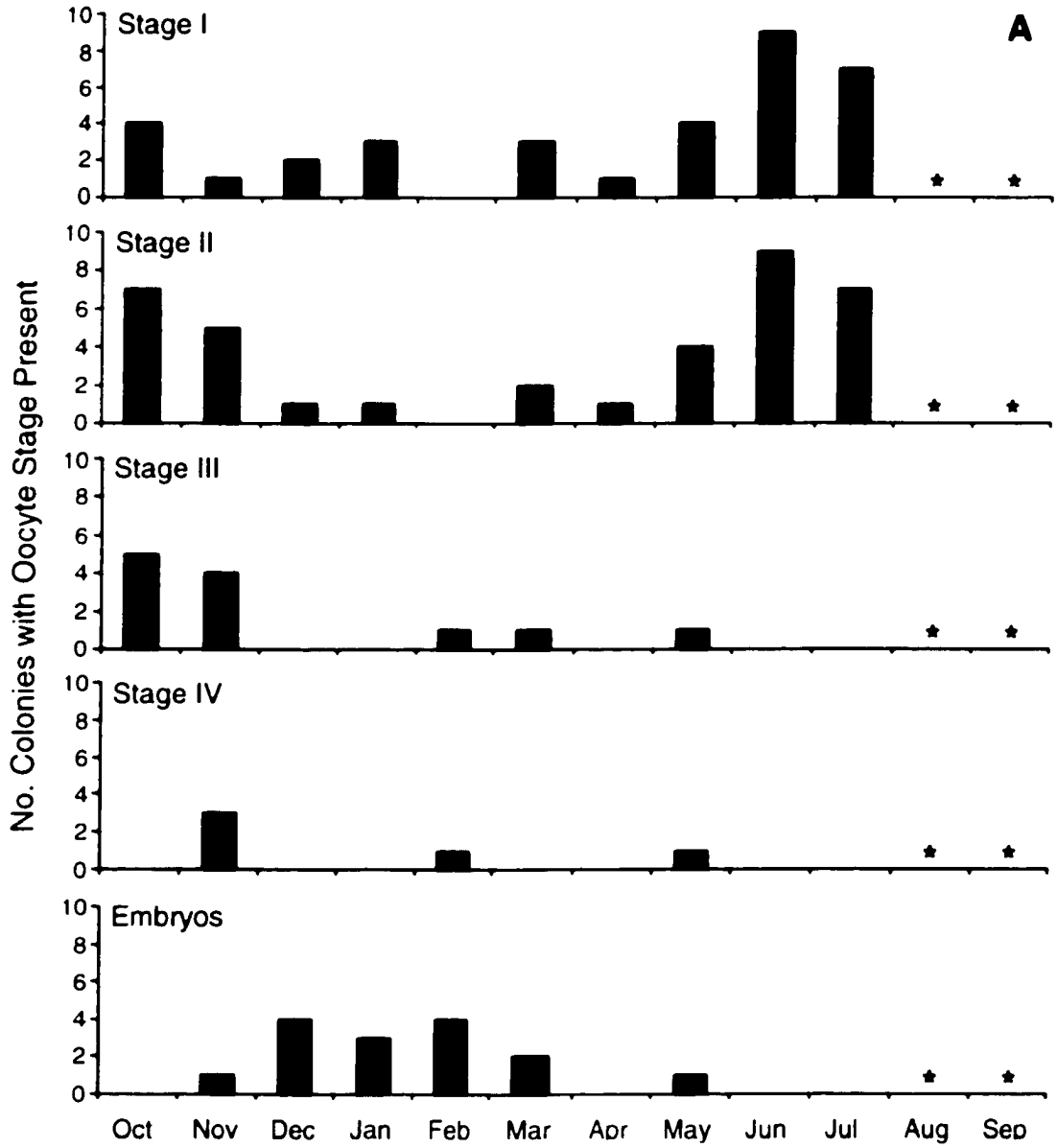

Figure 4.4: Presence of oocytes and embryos in ten *Halichondria* spp. colonies per month at each study site, October 1996-September 1997. A. *H. panicea* at Outside Beach. Asterisk (*) = no data, no samples collected.

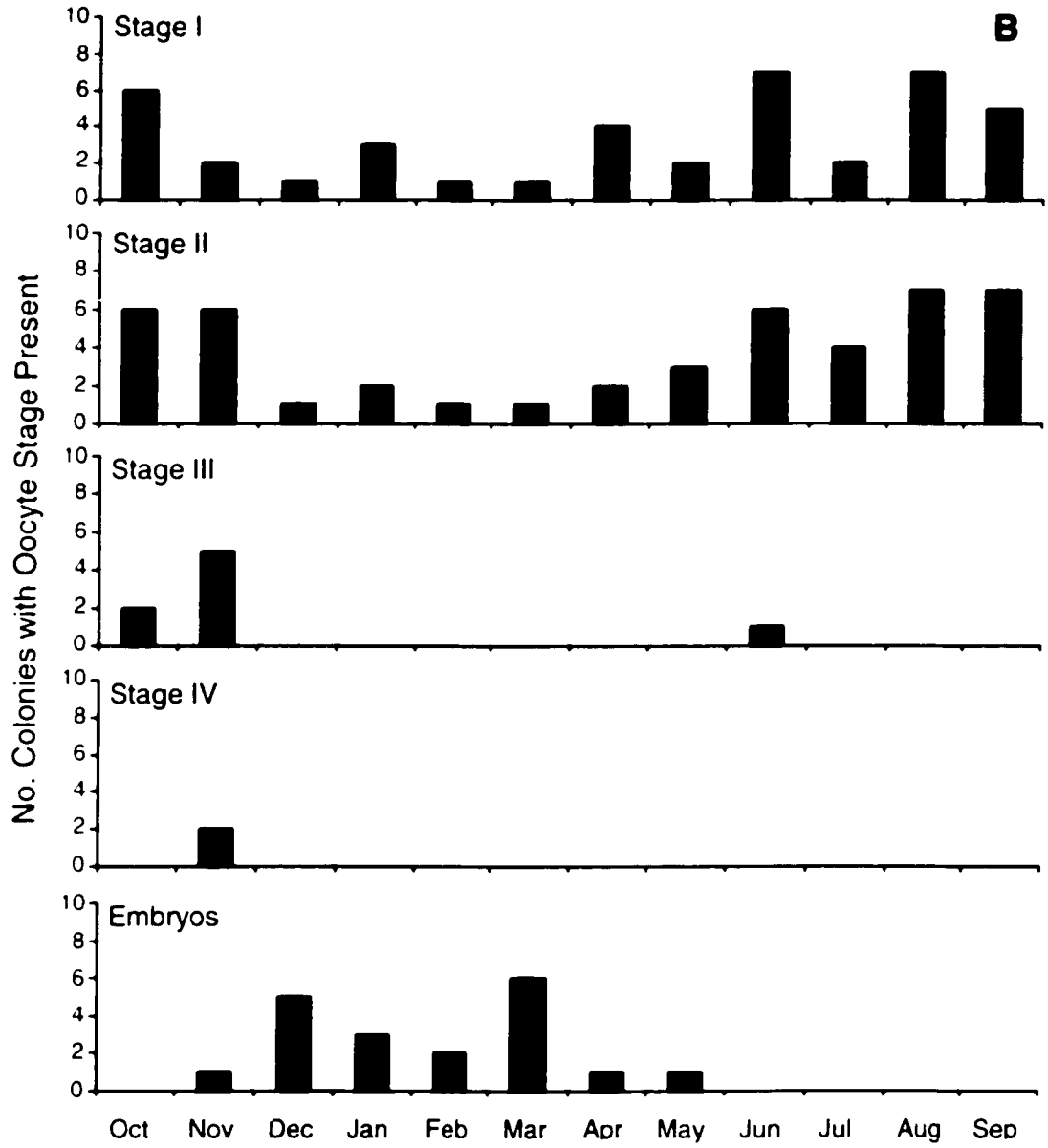

Figure 4.4B: *H. panicea* at Camel Rock.

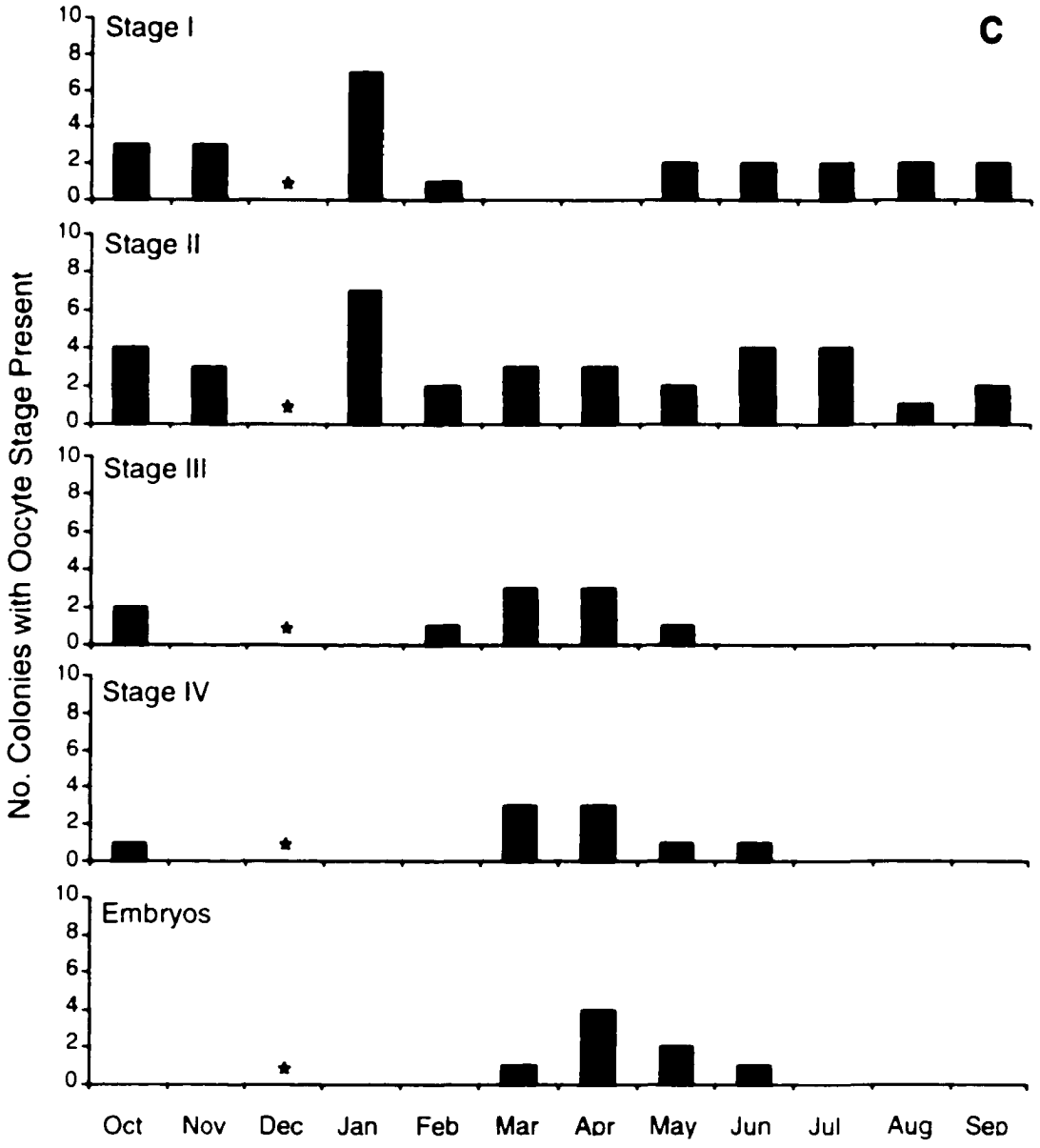

Figure 4.4C: *H. bowerbanki* at Jakolof Bay. Asterisk (*) = no data, no samples collected.

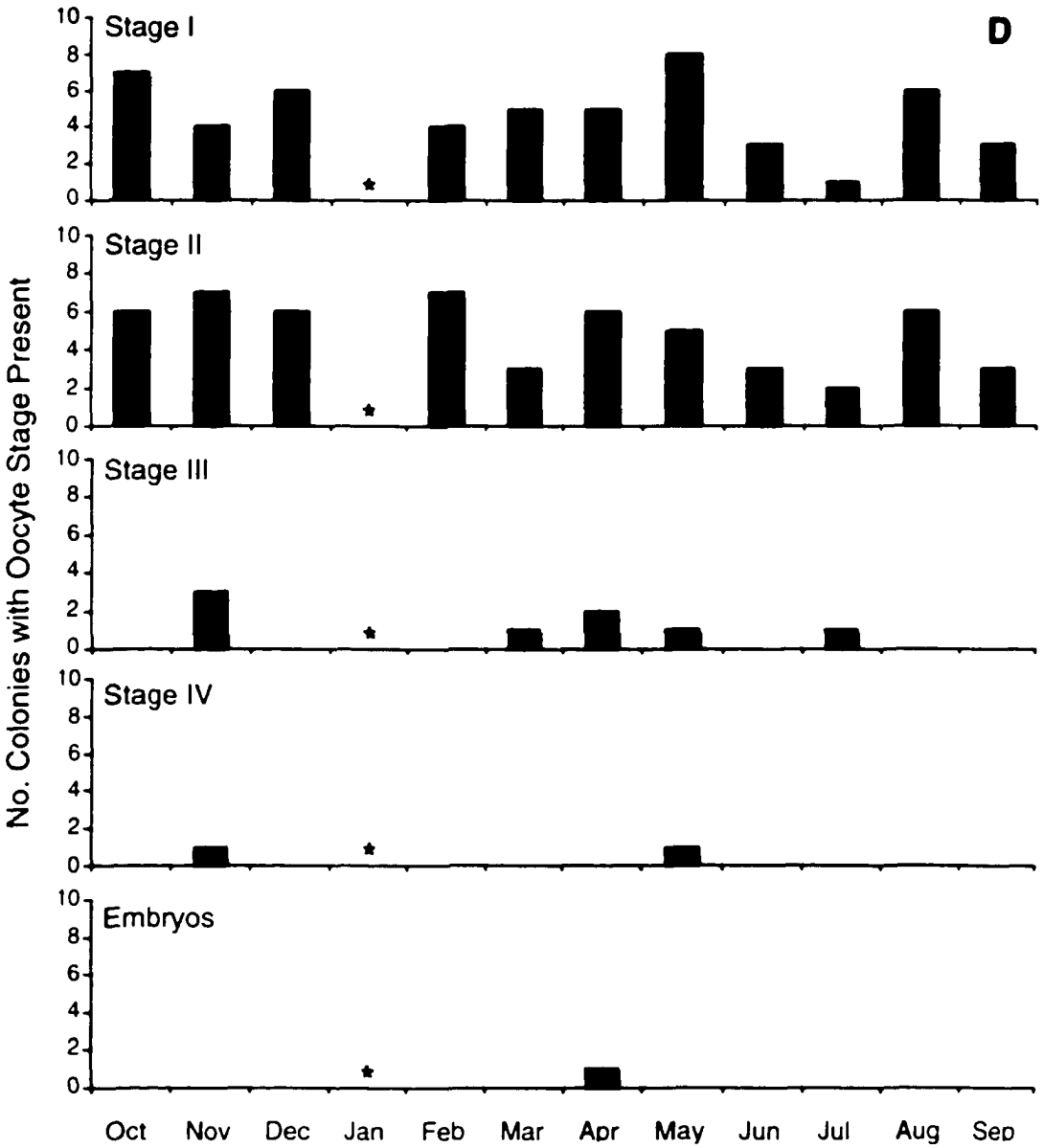

Figure 4.4D: *H. bowerbanki* at Seldovia Bay. Asterisk (*) = no data, no samples collected.

could have been missed by chance due to timing of sample collection and selection of colonies to be sampled. In addition, not all of the small oocytes will mature at the same time; a portion are retained throughout the year, decreasing the number that will mature into large oocytes.

Sex Ratios

The majority of individuals sampled from all populations contained female reproductive characteristics (e.g. oocytes, embryos) and were categorized as females (Table 4.4). Those samples containing only spermatocysts were considered males. A few individuals contained both male and female structures (e.g. oocytes and spermatocysts) and were scored as simultaneous hermaphrodites. In addition, reproductive structures were not detected in some samples which were therefore listed as non-reproductive. Overall male:female sex ratios, excluding hermaphrodites, ranged from 1:3 to 1:8 (Table 4.4). No habitat-related pattern was observed.

Oocyte Density

To quantify oocyte production, which is proportional to fecundity, oocyte density for samples containing stage I through stage IV oocytes was calculated (Table 4.5). Within each sampling date and site, oocyte density was measured as the number of oocytes per cubic millimeter of sponge tissue. For nearly all collection dates at a site, there was high variability in oocyte densities between

Table 4.4: Number of *Halichondria* spp. colonies across time by reproductive state in different habitats, Kachemak Bay, Alaska, October 1996-September 1997. Sex ratios were determined using only male and female colonies and were rounded to the nearest whole number.

	Halichondria panicea		***Halichondria bowerbanki***	
	Outside Beach	Camel Rock	Jakolof Bay	Seldovia Bay
Female	49 (49%)	58 (48%)	38 (35%)	58 (53%)
Male	18 (18%)	7 (6%)	9 (8%)	7 (6%)
Hermaphrodite	7 (7%)	9 (8%)	4 (3%)	2 (2%)
Non-Reproductive	26 (26%)	46 (38%)	59 (54%)	43 (39%)
Total	100	120	110	110
M:F Sex Ratio	1:3	1:8	1:4	1:8

Table 4.5: Monthly oocyte densities (oocytes/mm^3 tissue) by site, October 1996-September 1997. Mean ± standard error, with n = the number of colonies containing developing oocytes. Embryos were not included. na = not available, no samples collected.

	Outside Beach	Camel Rock	Jakolof Bay	Seldovia Bay
October 1996	7.88±2.87 n = 7	6.58±3.41 n = 7	2.64±1.96 n = 4	3.37±1.93 n = 7
November 1996	20.72±9.90 n = 5	9.40±4.19 n = 5	3.52±2.58 n = 4	2.71±1.66 n = 7
December 1996	4.24±3.30 n = 3	2.00 n = 1	na	6.13±2.51 n = 7
January 1997	0.75±0.59 n = 3	17.77±8.67 n = 3	51.23±10.38 n = 7	na
February 1997	0.32 n = 1	22.98 n = 1	22.34±22.30 n = 2	5.19±3.38 n = 7
March 1997	9.22±8.67 n = 4	33.16 n = 1	50.00±23.17 n = 3	9.11±7.04 n = 5
April 1997	0.17 n = 1	0.89±0.46 n = 5	14.27±12.23 n = 4	7.45±3.67 n = 6
May 1997	1.44±0.34 n = 4	1.47±0.72 n = 3	0.34±0.02 n = 3	0.73±.032 n = 5
June 1997	2.16±0.72 n = 9	2.41±1.22 n = 7	0.55±0.37 n = 3	0.76±0.28 n = 3
July 1997	1.72±0.31 n = 7	0.18±0.06 n = 4	3.58±3.49 n = 4	10.29±10.23 n = 2
August 1997	na	3.86±1.74 n = 7	0.35±0.32 n = 2	2.89±2.04 n = 6
September 1997	na	4.44±2.22 n = 7	2.21±1.87 n = 2	5.64±5.50 n = 4

colonies. This lack of synchrony probably accounts for some of the scatter in Figure 4.4.

Discussion

Genetic or environmental differences could account for the winter vs. spring late-stage reproductive events in *Halichondria panicea* and *H. bowerbanki*, respectively, in Alaska. Studies in the Netherlands (Vethaak et al., 1982; Wapstra and van Soest 1987) illustrate a similar difference in reproduction for the two species with both intertidal and subtidal populations of *H. bowerbanki* lagging behind *H. panicea* populations by 1-2 months (Table 4.6, 4.7).

Prior studies on *Halichondria panicea* found that production of large oocytes and embryos occurred in late-summer rather than during winter-early spring as in the Alaska populations and only one other study found small oocytes all year long (Table 4.6). One would expect sponge populations in Alaska to behave more like the Barents Sea population than any of the Atlantic populations because both sampling sites were high latitude, intertidal locations. Timing of reproductive events at the two locations was not the same. Probable causes for the difference include influence of warm Gulf Stream waters in the Barents Sea, even though it is part of the Arctic Ocean, and potentially colder air temperatures in the Barents Sea that may delay intertidal sponge reproductive activities. The intertidal Alaska populations most resemble the subtidal Baltic Sea *H. panicea*

Table 4.6: Reproductive activity of *Halichondria panicea* populations at various localities in the Northern Hemisphere based on literature and this study. so = small oocytes, lo = large oocytes, o = oocytes, e = embryos, l = larvae, nr = no reproductive structures observed. Shaded cells indicate months not included in the referenced study or months where sample collection was not indicated.

Location	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Reference
Alaska[1]	so lo	so lo e	so lo e	so lo e	so lo e	so lo e	so lo e	so lo e	so	so	so	so	This study
Baltic Sea[2]	so	so	so	so	so	so lo e	so lo e	so lo e	nr	nr	nr	nr	Witte & Barthel 1994
Netherlands[2]	o	o	o	o	o	o	o	o e	o e l	o e l	o e l	o e l	Wapstra & van Soest 1987
Netherlands[1,2]	nr	nr	nr	nr	nr	nr	nr	lo e	lo e	lo e	lo e	nr	Vethaak et al 1982
California[2]								lo e	lo e	lo e	lo e		Fell 1974
Barents Sea[1]	e	nr	nr	so	so	so	so	so	so	so lo	so lo e	e	Ivanova 1981

[1]intertidal
[2]subtidal

Table 4.7: Reproductive activity of *Halichondria bowerbanki* populations at various localities in the Northern Hemisphere based on literature and this study. so = small oocytes, lo = large oocytes, o = oocytes, e = embryos, l = larvae, nr = no reproductive structures observed. Shaded cells indicate months not included in the referenced study.

Location	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Reference
Alaska[1,3]	so	so	so	so	so lo	so lo e	so lo e	so lo e	so lo e	so	so	so	This study
Netherlands[2]	o e l	o e l	nr	nr	nr	nr	o	o	o e	o e l	o e l	o e l	Wapstra & van Soest 1987
Netherlands[1,2]	lo e	nr	nr	nr	nr	nr	nr	nr	nr	nr	lo e	lo e	Vethaak et al 1982
Connecticut[2,4]							so	so lo e l	so lo e l	so e l	nr	nr	Fell & Jacob 1979

[1]intertidal
[2]subtidal
[3]species complex of *H. bowerbanki* and *Halichondria*-like species
[4]based on *Halichondria* sp. that closely resembles *H. bowerbanki*

populations in their reproductive activity though differences are apparent. Maturation of oocytes and the appearance of embryos begin earlier in Alaska and continue over a longer time period than in the Baltic Sea. While located in separate oceans, Alaska and Baltic Sea sponge populations may experience similar environmental conditions. The Baltic Sea is also influenced by warm waters from the Gulf Stream, but as the populations studied were subtidal, they would experience less environmental variation than an intertidal population. The northern Gulf of Alaska has a similar warm water influence from the North Pacific Current, though it is mitigated to an extent by coastal upwelling.

The reproductive cycle of *Halichondria bowerbanki* has been studied less extensively (Table 4.7). As with *H. panicea*, *H. bowerbanki* populations in Alaska contained large oocytes and embryos earlier in the year than observed in other populations. The Connecticut population is most similar in timing of reproductive events, but occurred 2-3 months later than in Alaska. Delay in the Connecticut population relative to the Alaska populations may be related to colder winter ocean temperatures and harsher winter weather along coastal Connecticut than typically experienced in Alaska. Spring phytoplankton blooms may occur later along the Connecticut coast, delaying growth and energy accumulation of the sponge colonies.

Factors potentially responsible for differences observed between the Alaska populations and other northern populations include regional productivity differences and sampling effort. Kachemak Bay is constantly flushed with

nutrient-rich waters (Sambrotto and Lorenzen 1986) and surface waters are well mixed (Burbank 1977). These conditions provide abundant food for sponges all year and allow them to be active during the winter instead of going into a dormant phase, as seen at other localities (Fell and Jacob 1979; Vethaak et al., 1982). Thus, more energy may be available for sustained reproductive efforts in Alaska. Sampling effort between the present study and others varied and may account for the increased observations of reproductive elements in this study which looked at 10 individuals from each site every month, with few exceptions. While most other studies did collect samples during the winter, fewer individuals were sampled and/or sampling was approximately monthly. Also, a larger portion of tissue was analyzed by light microscopy in this study than in others thereby increasing the chance of finding oocytes and embryos.

At the initiation of this study, it was thought that a single species, *Halichondria panicea*, occurred in both habitat types. However, subsequent molecular work (Chapter 5, 6) revealed high levels of genetic differentiation among individuals in different habitat types and that the same species did not occur in both exposed and sheltered habitats. Thus, habitat effect on the timing and strength of reproductive activity could not be determined independent of genetic differences. Comparisons with previously studied populations in varying habitats did not clearly reveal habitat effects, perhaps because most studies were on northern subtidal populations. Differences between intertidal and subtidal conditions, greatly separated geographic locations, and varying baseline

environmental factors make broad comparisons difficult. Also, the accuracy of species identifications may vary by location. The question of habitat effect on life histories is important and should be investigated, but a single, well-identified and broadly distributed species is required.

Prior studies reported male:female sex ratios varying from 1:2 to 1:10 in *Halichondria panicea* populations (Witte and Barthel 1994; Witte et al., 1994). The overall ratios in the present study were also skewed towards females with ratios of 1:3 and 1:8 for the two *H. panicea* populations (Table 4.4). Similar ratios were observed for the *H. bowerbanki* populations. Female colonies may be detectable over a longer period of time than male colonies. Oocytes generally take several months to develop and mature while it only takes a few weeks for sperm to mature (Wapstra and van Soest 1987; Witte et al., 1994). Otherwise, this widespread imbalance in sex ratios is hard to explain. Male colonies did not appear to be seasonally limited, as individuals were found throughout the study period in both habitats. However, the majority of males (16 of 25 at exposed sites and 9 of 16 at protected sites) occurred in the month prior to the first appearance of embryos within each habitat and the two months following. The presence of male individuals prior to and during the early time period of embryo occurrence is logical as sperm would be required to fertilize mature oocytes for development into embryos.

There are conflicting reports on the extent of hermaphroditism in sponges. Witte and Barthel (1994) observed complete gonochorism, or separation of

sexes, in *Halichondria panicea* in the Baltic Sea, while Wapstra and van Soest (1987) reported a high degree of hermaphroditism in both *H. panicea* and *H. bowerbanki* in SW Netherlands. *H. panicea* exhibited simultaneous hermaphroditism while *H. bowerbanki* was a sequential hermaphrodite. The Alaska populations investigated contained a small number of simultaneous hermaphrodites, ranging from 2-8% of the total number of samples analyzed at each site (Table 4.4). There were no obvious seasonal patterns in the occurrence of hermaphrodites. Because individual colonies were not tagged and repeatedly sampled, the true extent of hermaphroditism may be underestimated for the Alaska populations. The sampling scheme would not detect sequential hermaphrodites unless there was a transition period when both male and female reproductive elements were present, but these individuals would be erroneously categorized as simultaneous hermaphrodites.

The relative importance of sexual and asexual reproduction may vary among habitats or species for intertidal sponges in southcentral Alaska. *Halichondria panicea* populations at exposed rocky sites produced more large oocytes and embryos than *H. bowerbanki* populations in sheltered, soft-sediment habitats. Few oocytes at an advanced stage of maturation or small numbers of embryos indicate a low degree of sexual reproduction. The soft-sediment populations of *Halichondria bowerbanki* do not appear to be diminishing and have persisted at the study sites for many years. Asexual reproduction is a plausible explanation for the persistence of this species and may be the primary

means by which it propagates. Genetic studies are needed to assess the degree of clonality within populations and indicate the extent of successful recruitment of asexual vs. sexual offspring into the population. However, *H. panicea* in exposed rocky habitats allocates relatively more resources for the production of sexual gametes. While mechanical stresses that facilitate asexual reproduction via fragmentation are prevalent in this habitat, the ability of fragments to reattach at wave-washed sites may be low. *H. panicea* appears to rely on sexual reproduction as its primary method of propagation.

Summary

Two intertidal sponge species, *Halichondria panicea* and *H. bowerbanki*, in southcentral Alaska, were investigated. The reproductive cycle of two populations of *H. panicea* from semi-exposed rocky habitats and two populations of *H. bowerbanki* from protected, soft-sediment bays exhibited seasonal peaks in oocyte production and maturation. In Alaska, peaks in reproductive activity were observed earlier in the year (winter-early spring vs. summer) than for both intertidal and subtidal populations of the same species investigated at other localities in the Northern Hemisphere. Small oocytes were present throughout the year indicating a degree of reproductive investment at all times. Within Alaska, populations in exposed habitats produced embryos 3-4 months earlier than populations in protected habitats (November vs. March). Initially, when it

was thought a single sponge species occurred at all of the sites, differences in the physical environment (water motion, sedimentation rate, salinity, water temperature) of the habitat types were hypothesized to cause this apparent shift in reproductive activity. Subsequent genetic work (Chapter 5, 6) revealed that there were different species occupying the habitats and habitat effect could not be separated from genetic variation. Male:female sex ratios for all populations ranged from 1:3 to 1:8, coinciding with sex ratios reported for other *Halichondria* populations. A few individuals sampled were simultaneous hermaphrodites, containing both oocytes and spermatocysts and ranging from 2-8% of each sample population. Based on the frequency of occurrence of large oocytes and embryos, *H. panicea* populations at exposed habitats appear to rely on sexual reproduction as the primary mode of propagation, while *H. bowerbanki* populations in protected habitats may rely more on asexual modes of reproduction (fragmentation, budding). Reproductive characteristics of intertidal populations of *H. panicea* and *H. bowerbanki* in southcentral Alaska contribute to our understanding of environmental influences on timing and mode of reproduction in sponges.

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Chapter 5: Genetic Structure of Intertidal Sponge Populations in Kachemak Bay, Alaska, Using Multiple Microsatellite Loci[1]

Introduction

Understanding dispersal and recruitment capabilities of species is a goal of much ecological research. Determining the genetic structure of populations, though difficult, is one approach to understanding population dynamics. In the past two decades, advances in molecular techniques and technology have allowed researchers to characterize organisms beyond outward morphological and ecological traits with increasing ease. Estimations of gene flow can indicate how intimately populations of a single species interact with one other.

Sponges are a challenging group to study, as taxonomic and phylogenetic relationships within the phylum are often unresolved. Identification of individual species can also be problematic due to plasticity and overlap of morphological characteristics. As a result, few studies have examined the population genetics of marine sponge species. To date, most studies of sponge population genetics (Solé-Cava and Thorpe 1986, 1991; Solé-Cava et al., 1991, 1992; Bavestrello and Sarà 1992; Boury-Esnault et al., 1992, 1999; Muricy et al., 1996; Miller et al.,

[1]Knowlton, A.L., S.L. Talbot, B.J. Pierson, and R.C. Highsmith. In preparation. Genetic structure of intertidal sponge populations in Kachemak Bay, Alaska, using multiple microsatellite loci. *Molecular Ecology*.

2001) have utilized allozyme assays, a technique that requires freshly collected tissues (Solé-Cava and Thorpe 1987). With advancing knowledge and technology, new genetic markers have been identified and utilized. Microsatellite markers are considered to be the tool of choice for investigating relationships between populations, due to their high levels of polymorphism and presumptive selective neutrality (McDonald and Potts 1996, Goldstein and Schlötterer 1999). However, taxon-specific markers need to be isolated and characterized.

The purpose of the research was to relate reproduction and dispersal modes and capabilities of the sponges to habitat occupation and abundance. Determining the degree of cloning occurring in each population would indicate the relative importance of sexual vs. asexual reproduction. A habitat effect was predicted due to differences in environmental conditions (wave energy, storm surge, etc.) that could also affect the likelihood of asexual reproduction. It was hypothesized that the degree of cloning would be higher for populations living in hard substrate habitats than for populations at soft-sediment sites, because of the greater opportunity for portions of sponge colonies to be separated or detached from the substrate and still remain at the same population site. Thus, genetic variability within hard substrate populations should be lower than for soft-sediment populations. Another issue of interest was potential gene flow between populations. Mixing and circulation patterns in the study area, Kachemak Bay, Alaska, are dominated by strong tidal currents (Burbank 1977). Therefore, it was hypothesized that local sponge populations would be panmictic, i.e. that there

was a free exchange of genetic material among all populations. For this study, the genetic structure of four intertidal sponge populations was examined. Two of the populations were from exposed, hard-substrate habitats and the other two were from protected, soft-sediment sites. Six polymorphic microsatellite loci were isolated and characterized for this purpose (see Appendix A).

Materials and Methods

Sample Collection

Tissue samples were collected from sponge colonies at four study sites along the southern shore of Kachemak Bay, Alaska (Fig. 5.1). Two sites, Camel Rock and Point Pogibshi, are semi-exposed hard substrate locations. The other sites, Jakolof Bay and Seldovia Bay, are protected soft-sediment habitats.

Finger-sized sections of tissue from 22-25 separate colonies were collected at each site and preserved in 100% ethanol. Genetic analyses were conducted at the U.S. Geological Survey's Molecular Ecology Laboratory in Anchorage, Alaska.

Genomic DNA Extraction

Genomic DNA was isolated from the sponge tissue samples following a CTAB/PVP (hexadecyltrimethylammonium bromide/polyvinylpyrrolidone) protocol

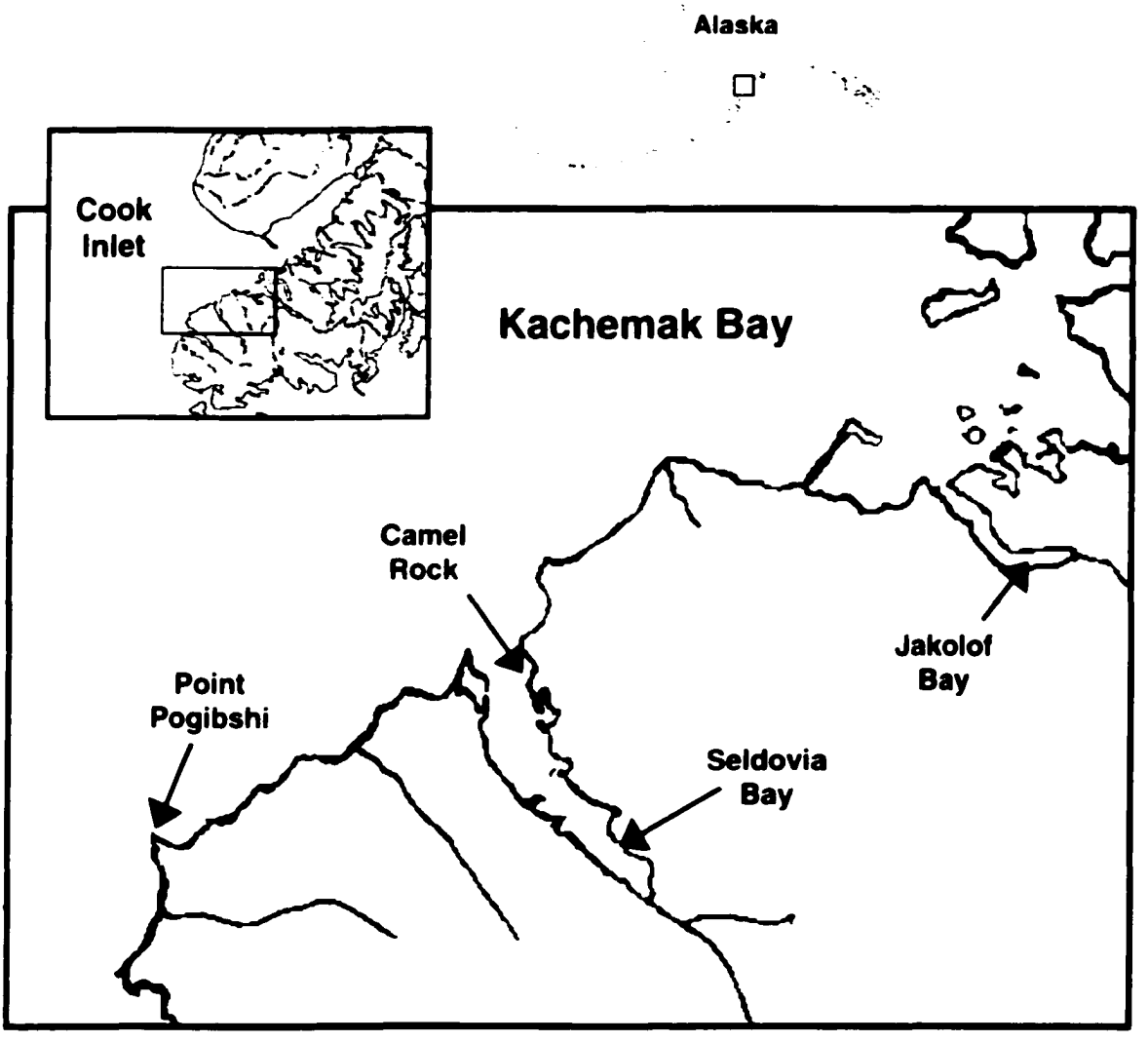

Figure 5.1: Location of sample populations in Kachemak Bay, Alaska, with regional context. Camel Rock and Point Pogibshi are exposed, hard-substrate habitats while Jakolof Bay and Seldovia Bay are protected, soft-sediment sites.

modified from Stewart and Via (1993). A 0.02-0.04 g portion of tissue was removed from the samples, minced, and placed in a 1.5-ml microcentrifuge tube. Each tube had 850 µl CTAB/PVP extraction buffer added with a brief mixing. To digest the tissue 5.5 µl Proteinase K (20 mg/ml) and 3 µl 2-mercaptoethanol were added and the tubes incubated with agitation at 55°C for 1-5 days or until the tissue was completely lysed. One hour prior to the end of incubation, 5 µl RNase (10 mg/ml) was added to remove any strands of RNA from the sample. The samples were centrifuged for 2 minutes at 5,000 rpm and the supernatant transferred to a clean tube. To remove mucopolysaccharides and proteins, 600 µl cholorform:isoamyl alcohol (24:1 v/v) was added to each tube and the samples vortexed at 500 rpm for 5 minutes followed by a 5-minute spin at 6,000 rpm. The upper aqueous layer containing the extracted DNA was transferred to a clean tube, mixed with 0.7 volume isopropanol, and allowed to precipitate overnight in a freezer. The purified DNA was pelleted at the bottom of the tubes by centrifuging for 5 minutes at 13,000 rpm. The alcohol was decanted and the DNA pellets allowed to dry overnight at room temperature. The DNA extractions were rehydrated with 30 µl sterile 1X TE (Tris EDTA) buffer and quantified using fluorometry. Each extraction was subaliquoted to make standard working stocks (50 ng/µl) for use in polymerase chain reaction (PCR) experiments.

Microsatellite Markers

Primer development. Fragments 400-1500 base pairs (bp) long and rich in CA- and GA-repeats were isolated and enriched from sponge genomic DNA. The fragments were ligated with a known vector and inserted into the circular genome of competent *Escherichia coli* cells. The bacterial cells were cultured overnight and a colorimetric indicator created within the vector sequence indicated which cell populations (colonies) successfully incorporated the vector sequence. Each positive colony was sequenced using primers made to attach to the known vector sequence. Analysis of the sequences showed the number of successful insertions of sponge DNA fragments along with the vector insertions. Not all successful sponge DNA insertions had microsatellite repeats in their sequences. Of the 56 positive colonies sequenced, eight colonies contained microsatellite repeats. Repeat motifs ranged from the targeted dinucleotide repeats to more complex repeat units. Primer sequences flanking the targeted repeat units were identified from the DNA sequences and sent to a private molecular service and supply company (QIAGEN Operon) for custom synthesis. A more detailed protocol can be found in Appendix A.

Data collection. DNA samples were genotyped at 6 of the 8 polymorphic microsatellite loci developed. Approximately 50 ng of DNA extract were amplified in a 10 µl PCR-reaction containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatin, 0.1 µg/µl bovine serum albumin (Hapa24), 200 µM

of each dNTP, 0.5 units of *Taq* polymerase, 0.1 μM M13 universal primer (Hapa21, Hapa28, Hapa37, Hapa46) or 0.15 μM (Hapa22, Hapa24), and 0.5 μM of each forward and reverse primer (Hapa24) or 1.0 μM (Hapa 21, Hapa22, Hapa28, Hapa37, Hapa46). The thermocycling profile consisted of a 2 minute denaturation at 94°C, followed by 30-40 cycles of 15 sec at 94°C, 15 sec at 50°-56°C (depending on the locus) and 30 sec at 70°C. An extension period of 30 minutes at 70°C concluded each thermocycler program. The PCR-products were separated on a 25 cm, 6% denaturing polyacrylamide sequencing gel and visualized using a LI-COR Longread 4200 automated sequencer (Lincoln, NE). Individual standards of known genotype obtained by sizing against an M13 sequencing reaction were run adjacent to the samples to provide an unambiguous size marker for the microsatellite alleles. A negative control was included in each reaction.

Statistical Analyses

All microsatellite loci were tested for gametic phase genotypic disequilibrium (for all two-locus comparisons) and for deviations from Hardy-Weinberg Equilibrium (HWE) (for each locus and for each population overall), using Fisher's exact test in GENEPOP 3.2a (Raymond and Rousset 1995). All p-values were adjusted for number of statistical tests (Sokal and Rohlf 1997), and an *a priori* condition was set that any loci found to be significantly linked due to gene geography were excluded from subsequent population-level analyses.

Observed (H_O) and expected (H_E) heterozygosity, mean number of alleles per locus (A) and allele size variance were estimated using Microsatellite Toolkit (Park 2000). Allelic richness, $r_{(g)}$, which corrects A for disparity in sample sizes was estimated (Petit et al., 1998). Estimates of H_O and H_E were used to generate inbreeding coefficients ($F=1-[H_O/H_E]$) combined across loci for each population and tested for significance as described in Li and Horovitz (1953). Values of the inbreeding coefficient range from 0.0 to 1.0. When F = 0, no inbreeding is occurring and genotypes are present in expected frequencies based on random mating models. If F = 1, complete inbreeding is occurring and the population consists of all homozygotes.

Gene frequencies were estimated for each microsatellite locus. Levels of population differentiation, based on the distribution of alleles across populations, were examined using Fisher's exact test, based on a Markov chain adaptation of row-by-column contingency tables, as generated by GENEPOP (Raymond and Rousset 1995). Distributions of alleles and genotypes across populations were examined using a log-likelihood (G) based exact test (Goudet et al., 1996) implemented by GENEPOP. Multiple test significance was judged using Fisher's exact test method and/or by applying sequential Bonferroni procedures (Rice 1995).

Significance of spatial variation in allele frequency was assessed using F-statistics (Weir and Cockerham 1984). These measures can be viewed simply as variance components and they describe the apportionment of allelic variance

among individuals within populations (F_{IS}) and among populations (F_{ST}). Values of F_{ST} are summary statistics describing the extent of spatial variation among populations or population groupings, and range from 0.0 to 1.0. A value of 1.0 at a specific locus would imply that all populations are "fixed" for different alleles (ie. the total variance at that locus is segregated among populations). At the other extreme, a value of 0.0 implies all populations share the same alleles in equal frequency. Multilocus estimates of F_{ST} were obtained using the program FSTAT 2.9.1 (Goudet 2000). Estimates of interpopulational variance (F_{ST}) were derived using the program ARLEQUIN 2.0 (Schneider et al., 1997). Significance of F_{ST} values was based on random permutation tests (n = 1000), whereby alleles were randomly permuted between the two populations. A significant F_{ST} value implies that a significant portion of the total genomic variation of the specific locus is partitioned among populations.

F_{ST} values assume adherence to the infinite allele model of mutational change (Maruyama and Fuerst 1985). R_{ST}, an analogue of F_{ST} (Michalakis and Excoffier 1996), was also calculated and assumes a stepwise mutation model that is derived from variances in mean allele size and frequency in relation to sample size and is seen as a more conservative distance measure relative to F_{ST} (Slatkin 1995). Statistical significance of R_{ST} was tested in the same manner as F_{ST} described above. For both tests, p-values were adjusted using Bonferroni corrections (Sokal and Rohlf 1997). Multilocus estimates of the effective number of migrants (N_m) per generation over all locations were calculated using private

alleles (Barton and Slatkin 1986). Genetic distances among populations were estimated with Cavalli-Sforza and Edwards (1967) distance, D_{CE}, calculated in BIOSYS 1.7 (Swofford and Selander 1989).

Detection of Bottleneck

Populations that experience a recent reduction of effective population size, such as during a founder event, are expected to show a reduction in both number of alleles and levels of heterozygosity at polymorphic loci (Watterson 1984). However, allelic diversity is reduced much more rapidly than levels of heterozygosity (Nei et al., 1975; Denniston 1978; Maruyama and Fuerst 1985), observed heterozygosity being larger than expected if the population was at mutation-drift equilibrium. Two statistical tests, the sign test and the Wilcoxon test, were used to detect excess heterozygosity for polymorphic microsatellite loci as an indicator of recent bottlenecks in each population (Cornuet and Luikart 1996). The sign test determines if the proportion of loci with heterozygosity excess is significantly larger than expected at equilibrium, and the Wilcoxon test determines if the average standardized differences between observed and expected heterozygosities is significantly different from zero. These two statistical tests detect recent bottlenecks, using heterozygosity and allele frequency data for each of several loci, and require no data on historical population size of levels of genetic variation. Tests were conducted using the program BOTTLENECK (Cornuet and Luikart 1996, Luikart and Cornuet 1998),

under three models thought to represent the range of possible mutation modes generating polymorphism at microsatellite loci. These include the infinite alleles model (IAM), the stepwise mutation model (SMM) (Freimer and Slatkin 1996, Jarne and Lagoda 1996), and the two-phase model (TPM) (see DiRienzo et al., 1994) of microsatellite mutation. One thousand simulations were performed for each population.

Results

Six of the eight microsatellite markers developed were consistently and reliably resolved (Hapa21, Hapa22, Hapa24, Hapa28, Hapa37, Hapa46). These six loci were used for the genetic analyses of the two types of intertidal sponge populations.

Genetic differences between hard substrate and soft-sediment sponge populations were immediately obvious. Both hard substrate populations, Camel Rock and Point Pogibshi, produced clear banding patterns for nearly all colonies within one or two PCR optimization experiments for the six usable microsatellite loci. The soft-sediment populations, Jakolof Bay and Seldovia Bay, produced unambiguous allele bands for three loci, but not all individuals yielded a product. Several PCR optimization attempts failed to produce usable results for the remaining loci, suggesting the possibility of a more distantly related taxon inhabiting soft-sediment habitats. Further investigation of this finding is

presented in Chapter 6. Since data could not be obtained from a sufficient number of microsatellite loci, the soft-sediment populations were dropped from this part of the study. Population genetic analyses continued with the two hard substrate populations and are presented below.

None of the individuals sampled in the hard substrate populations were genetically identical for all the loci investigated. Measures of genetic diversity of the hard substrate populations are summarized in Table 5.1. Mean number of alleles per locus was 5.0 for Camel Rock and 6.3 for Point Pogibshi and allelic richness was 4.9 and 5.9, respectively. Point Pogibshi was observed to have 14 private, or non-shared alleles, while only 7 were observed for Camel Rock. Inbreeding coefficients were not significant for either population. Overall, Camel Rock was in Hardy-Weinberg Equilibrium (HWE) while Point Pogibshi was not. In a locus by locus examination, two loci per population were out of HWE (Table 5.2). Deviations from HWE often are a result of violations of the assumptions of the standard random-mating model and can indicate that structuring mechanisms (e.g. selection, mutation, migration, non-random mating, asexual reproduction) are influencing the distribution of alleles or genotypes within a population. A deficiency of heterozygotes often results in populations being out of HWE overall or for a specific locus (Hartl 2000).

The six microsatellite loci were characterized by population and over all samples (Table 5.2). No significant genotypic disequilibrium within or overall was detected. Thus, all loci appeared to be inherited independently of one another

Table 5.1: Measures of genetic diversity at six microsatellite loci for two populations of *Halichondria panicea*. Values reported are sample size (n), mean number of alleles per locus (A), allelic richness ($r_{(g)}$), number of private or non-shared alleles (PA), observed heterozygosity (H_O), expected heterozygosity (H_E; Nei 1987), and inbreeding coefficient ($F= 1-[H_O/H_E]$; Li and Horvitz 1953). Standard errors are in parentheses. Asterisk (*) indicates population not in Hardy-Weinberg Equilibrium.

Population	n	A	$r_{(g)}$	PA	H_O	H_E	F
Camel Rock	22	5.0 (1.0)	4.9 (1.0)	7	0.467 (0.089)	0.532 (0.113)	0.122
Point Pogibshi	25	6.3 (1.6)	5.9 (1.4)	14	0.493* (0.118)	0.603 (0.130)	0.182

Table 5.2: A description of six microsatellite loci and summary of allelic variation within two populations of *Halichondria panicea*.

			Camel Rock		Point Pogibshi	
Locus	**Alleles[1]**	**Size Range[2]**	**H_E[3]**	**P[4]**	**H_E**	**P**
Hapa21	8	167-243	0.778	7	0.749*	5
Hapa22	13	186-255	0.519	6	0.823	11
Hapa24	10	155-189	0.758*	8	0.762*	9
Hapa28	2	175-179	0.130	2	0.000[5]	1
Hapa37	3	257-289	0.274	2	0.494	3
Hapa46	9	200-234	0.733*	6	0.793	9

[1]Total number of alleles found within both populations studied
[2]Size range in base pairs, including primers
[3]Expected heterozygosity (Nei 1987; eq. 7.39, p.164). Asterisk (*) indicates locus out of Hardy-Weinberg Equilibrium for population.
[4]Total number of alleles found within population
[5]Population is monomorphic at this locus

and were retained for further analyses. Significant differences between populations were observed for several genetic characteristics. Genic, or allelic, differentiation was observed at four of the six loci (Hapa21, Hapa22, Hapa24, and Hapa37) and overall the populations were significantly different. Similar results were obtained for genotypic differentiation with three loci (Hapa21, Hapa22, and Hapa37) showing significant differences, as well as the populations being significantly differentiated from one another.

F-statistics describing variances in allele frequency between and within populations were calculated. Among populations, an F_{ST} value of 0.047 was significant, while an R_{ST} of 0.026 was not significant. Differentiation among individuals within populations was low (F_{IS} = 0.164). The number of migrants transporting genetic material between populations was sufficient to maintain geneflow (N_m > 2). The genetic distance between populations (D_{CE}) was 0.318.

For the three mutational models tested, no significant bottleneck effect was detected in either population (Table 5.3). None of the loci were significant for heterozygote excess relative to the number of alleles. One locus (Hapa24) had fewer heterozygotes than expected from HWE in the Camel Rock population, while three loci (Hapa21, Hapa22, and Hapa24) had significant heterozygote deficits for Point Pogibshi samples. This pattern is observedin rapidly expanding populations or when populations are recruiting immigrants from differentiated populations (Luikart and Cornuet 1998). One locus (Hapa28) was monomorphic at Point Pogibshi. Not all data analyses could be completed

Table 5.3: Sign and Wilcoxon tests for heterozygosity excess at six microsatellite loci in two populations of *Halichondria panicea*. The three mutational models used were the infinite alleles model (IAM), the two-phase model (TPM) and the stepwise mutation model (SMM). Significance was tested at the $\alpha = 0.05$ level and a Bonferroni correction was applied. e/d = ratio of heterozygote excess to deficit, N = number of alleles compared in a diploid population.

	Sign Test			**Wilcoxon Test**					
	Mutational Model			**Mutational Model**					
	IAM	**TPM**	**SMM**	**IAM**		**TPM**		**SMM**	
Population (N)	e/d	e/d	e/d	H excess	H deficit	H excess	H deficit	H excess	H deficit
Camel Rock (44)	4/2	3/3	2/4	0.422	0.656	0.656	0.422	0.078	0.039
Point Pogibshi (50)	4/1	2/3	2/3	0.031	0.984	0.406	0.688	0.953	0.078

for a fixed allele locus.

Discussion

One of the more revealing outcomes of this study was the inability to compare soft-sediment and hard substrate sponge populations. Historical accounts and identifications maintained that a single species, *Halichondria panicea*, predominated in the intertidal habitats of southcentral Alaska. Differences in outward appearances were attributed to a high degree of morphological plasticity exhibited by sponges in response to environmental factors, primarily wave action (Palumbi 1984). The genetic markers developed for this study were isolated from a single individual sponge colony from the hard substrate sponge population at Camel Rock. Presuming a single species resided at all study sites, genotypes should have been obtained for all populations under study. The fact that little data could be gathered from either soft-sediment population indicates a substantial genetic divergence between populations in different habitats. The results from the current study are insufficient to determine whether or not the divergence is great enough to classify the soft-sediment and hard substrate populations as separate species, but they do indicate that gene flow between populations in the two habitat types is extremely low to nonexistent. This question is addressed further in Chapter 6. The original hypothesis that the sponge populations in Kachemak Bay were panmictic is not supported. A barrier

to gene flow exists between soft-sediment and hard substrate sponge populations.

While analyses of soft-sediment populations could not be completed, genetic characterization of the hard substrate populations (Camel Rock and Point Pogibshi) was possible. Among all hard substrate samples, no two sponge colonies were genetically identical at the six loci examined. Based on this result from a broad survey of sponge colonies at each site, cloning, or asexual reproduction, is not a major factor in the persistence of the populations. In addition, it does not appear that asexual products exported from one site are retained at the other. Wave forces and tidal currents may be strong enough to minimize or prohibit the ability of a sponge fragment to reattach to the substrate within its source population.

Based on the frequency of intercolony graft rejection or acceptance, Jokiel et al. (1982) determined a high degree of cloning (5% to 23%) within highly localized sponge populations. Graft acceptance in colony pairings categorized the colonies as clonemates. Curtis et al. (1982) found discrepancies between intercolony graft acceptance and protein assays. Graft-accepting colony pairs did not necessarily show the same electrophoretic banding patterns, as would be expected if they were genetically identical. Molecular techniques analyzing an organism's DNA have been refined and can provide more appropriate data for answering the question of the relative importance of sexual vs. asexual reproduction in a population.

Genetic differences were observed between Camel Rock and Point Pogibshi. The distribution of alleles, genic differentiation, between the populations was significant meaning that some alleles were more frequently associated with one population than expected based on Hardy-Weinberg predictions. In addition, genotypic differentiation was significant for half of the loci examined. Specific genotypes, or groups of alleles, were more frequently associated with one population than expected. Both of these results indicate that differentiation is occurring between the hard substrate populations. F-statistics estimate the degree of divergence of populations. A significant F_{ST} value was obtained indicating that differentiation was occurring, but its low value (F_{ST} = 0.047) suggests that Camel Rock and Point Pogibshi have diverged recently and are still closely related. An analogous statistic, R_{ST}, was not significant and this result also supports a more recent divergence of the populations. R_{ST} is a better estimator of differentiation for populations that are more distantly related, where mutation is the most important force causing genetic change, while F_{ST} produces better estimates for recently diverged populations where genetic drift and migration are the primary sources of genetic variation.

Placing the results of this study in context with other population genetics investigations of sponges and clonal organisms provides a broader reference point for genetic variability found in benthic marine invertebrates. A comparison of F_{ST} values shows that the variability among sponge populations in this study is within the range of observed variability for other sponge species, as well as other

clonal invertebrates (Table 5.4). However, all of the other studies utilized allozyme (protein) assays while this study examined DNA microsatellite loci. One would expect a higher level of variability when examining actual genes as opposed to gene products. The hard substrate populations in this study experienced similar environmental conditions and were in relatively close proximity to each other. Both factors could decrease the observed variability between populations. Results from studies utilizing microsatellite loci would be more appropriate comparisons, but have yet to be completed for other sponge populations.

Summary

The genetic population structure of four intertidal sponge populations, two from exposed, hard-substrate habitats and two from protected soft-sediment sites, was determined using microsatellite loci. Six variable and reliable microsatellite regions were isolated and characterized from a single sponge colony collected at a hard substrate site. Results of the genetic analyses showed striking differences between habitat types. Samples from the soft-sediment locations could only be amplified at three of the six loci. The inability to amplify soft-sediment samples with the microsatellite markers suggests significant genetic differentiation from the hard substrate populations. The soft-sediment populations were dropped from further investigation, but analyses continued for

Table 5.4: Comparison of F_{ST} values for several sponge species and other clonal marine invertebrates.

Species	Genetic Analysis	F_{ST}	Study Location	Reference
Porifera				
Carterospongia flabellifera	allozyme	0.360	NE Australia	Benzie et al.,1994
Collospongia auris	allozyme	0.050	NE Australia	Benzie et al.,1994
Halichondria panicea	microsatellite	0.047	Alaska	This Study
Halisarca laxus	allozyme	0.013	SE Australia	Davis et al.,1996
Phyllospongia alcicornis	allozyme	0.240	NE Australia	Benzie et al.,1994
Phyllospongia lamellosa	allozyme	0.230	NE Australia	Benzie et al.,1994
Urochordata				
Botrylloides magnicoecum	allozyme	0.202	SE Australia	Ayre et al.,1997
Stolonica australis	allozyme	0.210	SE Australia	Ayre et al.,1997
Cnidaria				
Antipathes fiordensis	allozyme	0.046	New Zealand	Miller 1997
Briareum asbestinum	allozyme	0.042	Caribbean	Brazeau & Harvell 1994
Zoanthus coppingeri	allozyme	0.039	NE Australia	Burnett et al.,1995

the hard substrate populations.

Characterizations of genetic diversity were completed for the hard substrate populations. Based on F_{ST} values, the hard substrate sponge populations are significantly differentiated from one another, but the low value of F_{ST} and a non-significant R_{ST} value suggest a more recent coalescence time. No significant inbreeding has occurred within either population and a bottleneck effect was not detected. None of the samples were genetically identical for the loci examined indicating that a low degree of cloning, or asexual reproduction, is occurring. Products of sexual reproduction appear to be maintaining the populations at their respective locations, though there is little gene flow between the populations.

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Chapter 6: Molecular Systematics of Sponges in Kachemak Bay, Alaska, Based on ITS Sequence Data[1]

Introduction

Taxonomic identification of Porifera is highly problematic because of overlapping morphological and ecological characteristics of many taxa. Morphological plasticity driven by environmental forces (e.g. water motion) acts to converge phenotypes, particularly among closely related groups. Within the order Halichondrida, species identification is especially challenging due to the simplicity and uniformity of spicules (Levi 1957), the primary identifying characteristic of sponges. Other morphological characteristics, such as growth form, color, spicule organization and density, and surface texture, have been coupled with biochemical and ecological data to revise the groupings found within Halichondrida (van Soest et al., 1990; Diaz et al., 1991; Pomponi et al., 1991). With the development of new molecular techniques, phylogenetic studies have utilized nuclear DNA sequence data from rRNA coding regions and other loci to analyze relationships among genera within families (see review, Borchiellini et al., 2000). Concurrent with the study reported here, Erpenbeck et

[1]Knowlton, A.L., S.L. Talbot, and R.C. Highsmith. In preparation. Molecular systematics of sponges in Kachemak Bay, Alaska, based on ITS sequence data. *Molecular Ecology.*

al., (2002) utilized a partial fragment of the cytochrome oxidase 1 (CO1) gene found in mitochondrial DNA to decipher phylogenetic relationships among halichondrid genera. While these DNA markers are useful at the genus level and above, more variable loci are needed to determine relationships between species. The internal transcribed spacer (ITS) regions located between the rRNA coding units are more variable than the rRNA regions and researchers have demonstrated the utility of the ITS regions for distinguishing closely related species (Beauchamp and Powers 1996; Chu et al., 2001; Patti and Gambi 2001; Chen et al., 2002).

Based on recent research (see Chapter 5), multiple taxa of *Halichondria* seem to inhabit Kachemak Bay, Alaska, where previously a single species, *Halichondria panicea*, was considered to occur. Results of population analyses (Chapter 5) suggest that the taxa are separated by habitat type, hard-substrate vs. soft-sediment. Based on morphological and ecological characteristics, the species most likely to occur in Kachemak Bay are *H. panicea* at exposed, hard substrate habitats and *H. bowerbanki* in protected, soft-sediment locations (Vethaak et al., 1982).

This study was initiated to determine the *Halichondria* taxa present in four sampled populations in Kachemak Bay. DNA sequence data from ITS and CO1 regions were utilized to determine the relationship of the sponge taxa among populations and within the context of other known species.

Materials and Methods

Sample Collection and Extraction

Sponge samples collected for the population genetics analyses (see Chapter 5) were also utilized for systematics investigations. Sufficient DNA was initially extracted from each sample for both microsatellite and sequencing polymerase chain reaction (PCR) amplifications.

ITS Sequencing

Primer development. Sequences for the rRNA/ITS-1 and ITS-2 region were obtained from product generated via PCR using a single forward and reverse primer pair. The targeted region was approximately 700 bp long and encompassed a portion of the 3' end of the 18S rRNA subunit, ITS-1, 5.8S rRNA subunit, ITS-2, and a fragment of the 5' end of the 28S rRNA subunit coding region (Fig. 6.1). The forward primer (5'–GTCCCTGCCCTTTGTACACA–3') was developed by Adlard and Lester (1995) and has been successfully used on several sponge genera (Lopez et al., 2002). The reverse primer (5'–GTTAGTTTCTTTTCCTCCGCTT–3') was designed from sponge rRNA sequence deposited in GenBank (Accession #U65485, Odorico and Miller 1997) and has also been successfully utilized for multiple sponge taxa (Lôbo-Hajdu, *pers. comm.*). An M13R (5'–GGATAACAATTTCACACAGG–3') tail was added to

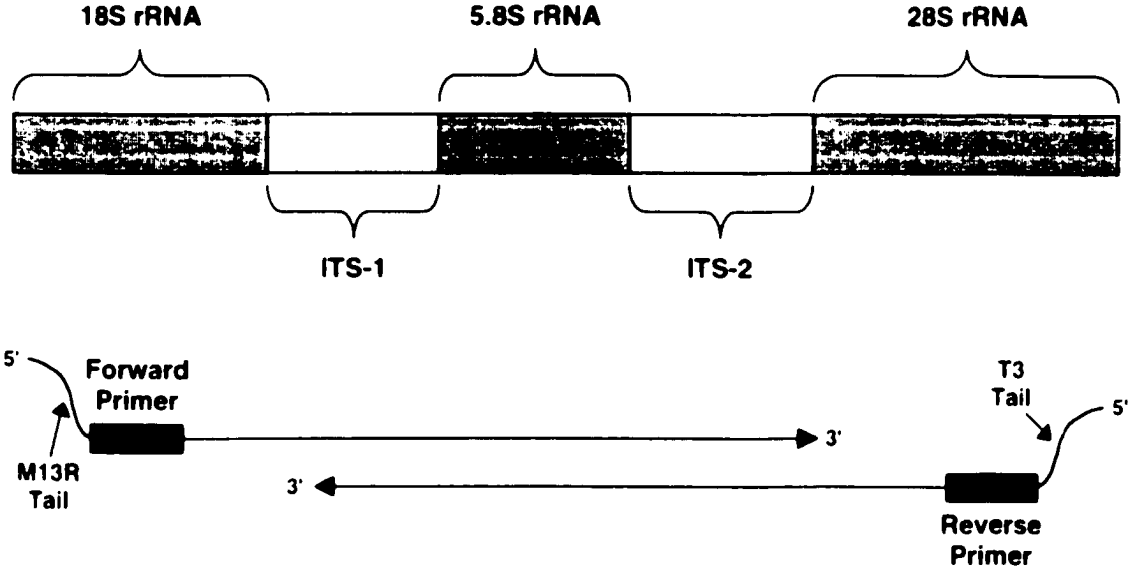


Figure 6.1: Diagram of the location of the internal transcribed spacers (ITS-1 and ITS-2) relative to the rRNA coding subunits on sponge nuclear DNA. Starting position and DNA elongation direction are noted for the forward and reverse primers. Length of fragment corresponds to approximately 700 base pairs. Diagram is not drawn to scale.

the 5' end of each forward primer; a T3 (5'-AATTAACCCTCACTAAAG-3') tail to each reverse primer (Steffens et al., 1993).

Data collection. Approximately 50 ng of DNA extract from each of 96 sponge colonies sampled were amplified in a 50 µl PCR-reaction containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatin, 190 µM of each dNTP, 1.25 units of *Taq* polymerase, and 1.0 µM each of the forward and reverse primers. The thermocycling profile consisted of a 2 minute denaturation at 94°C, followed by 40 cycles of 1 minute at 94°C, 1 minute at 50°C and 2.25 minutes at 70°C. PCR products were visualized on a 1.2% agarose gel. Reaction mixtures exhibiting appropriate size product bands were subsequently purified using Quantum Prep® PCR Kleen Spin Columns (BIO-RAD) and subjected to tailed primer bi-directional cycle sequencing reactions (Steffens et al., 1993) using SequiTherm EXCEL II™ DNA Sequencing Kits-LC. Conditions slightly modified from the manufacturer's recommendations and employing IRDye700 and IRDye800 labeled M13R and T3 primers, respectively, were used. Sequenced products were separated via electrophoresis on a 66 cm, 3.7% polyacrylamide (KB-Plus™, LI-COR, Lincoln, Nebraska) gel using a LI-COR LongReadIR™ 4200 two-dye automated sequencing system. Sequences were captured using LI-COR GeneImagIR™ Data Analysis software and aligned using Aligner™ computer statistical package on a Gateway computer.

CO1 Sequencing

Primer development. Sequences for a 500 bp long fragment of the mitochondrial DNA Cytochrome Oxidase I (CO1) region were obtained from product generated via PCR using a single forward and reverse primer pair. The forward (5'-TGATACCTACCTTTTCTGC-3') and reverse primers (5'-AAAGGTTAAATTCACAC-3') were designed from *Halichondria panicea* CO1 sequence deposited in GenBank (Accession #AF437294, Erpenbeck et al., 2002). An SP6 (5'-GATTTAGGTGACACTATAG-3') tail was added to the 5' end of the forward primer; a T3 (5'-AATTAACCCTCACTAAAG-3') tail to the reverse primer.

Data Collection. A small subset of sponge samples (n = 10) including at least two individuals categorized for each ITS haplotype were sequenced for a fragment of the CO1 region of mtDNA. The PCR reaction mixture and thermocycling conditions for the CO1 fragment were the same as for the ITS reactions, but utilized the CO1-specific forward and reverse primers. Purification and imaging of the resulting PCR products were performed in the same manner as for the ITS PCR products with the exception that IRDye700 and IRDye800 labeled T3 and SP6 primers, respectively, were used.

Sequence Analyses

Sequences were aligned using eSEQ (Version 2.0, LI-COR) and analyzed using both parsimony and distance methods. Analyses based on weighted parismony were performed using PAUP (Version 4*, Swofford 2000). A published ITS sequence of the sponge *Axinella corrugata* (Lopez et al., 2002) was used as an outgroup for ITS analyses. CO1 sequence data for several sponge species (Erpenbeck et al., 2002) were included in analyses of Alaska sponge CO1 sequences. The most parsimonious trees were obtained using the branch-and-bound option in PAUP. A majority rule consensus tree based on 1000 bootstrap replicates was produced using the bootstrap option in PAUP, which is based on the methods of Eck and Dayhoff (1966) and Kluge and Farris (1969) and on Fitch's (1971) method of counting the number of base changes needed on a given tree. Distance trees were produced using the neighbor-joining method of Saitou and Nei (1987) and the computer program MEGA2 (Version 2.1; Kumar et al., 2001). The Tamura-Nei (1993) model of DNA sequence evolution was used to generate distance matrices. Base compositions of the ITS regions were analyzed using MEGA2.

Population Genetics Analyses

Relationships among sites were determined by constructing neighbor-joining phylogeographic trees using coancestry coefficient distances (Reynolds et

al., 1983) generated from the AMOVA analysis of ARLEQUIN 2.0 (Schneider et al., 1997) and PHYLIP (Version 3.57c, Felsenstein 1993) software packages.

Results

ITS Sequences

Three major haplotypes, designated as ITS A, ITS B, and ITS C, were identified among 96 samples sequenced. A total of 55 variable sites were observed among the three haplotypes (Table 6.1). Variation among the sequences was characterized by pairwise comparisons, average of all pairwise comparisons, and overall sequence comparison (Table 6.2). The number of base insertions or deletions (indels) ranged from 8 to 15 sites with a total of 16 different indel sites. The number of transitional site changes (t_i), base changes between A↔G and T↔C, varied from 3 to 29 sites. Transversion substitutions (t_v) comprised all other base substitutions and ranged from 1 to 9 sites. The highest number of t_i and t_v sites were observed in comparisons with haplotype B. No multistate changes, i.e., more than two bases present at a specific site, were observed. The ratio of transitions to transversions (R) ranged from 3.0 to 3.6 for pairwise comparisons and averaged 3.3.

Parsimony analysis using the branch-and-bound method yielded a single parsimopnious tree (tree length = 32.833, CI = 0.970, RI = 0.966). The tree grouped haplotypes A and C together as a sister group relative to haplotype B

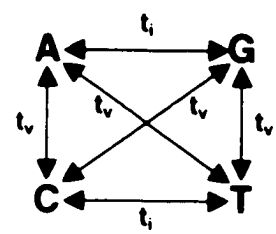
Table 6.1: Variable sites within the internal transcribed spacers (ITS) of three sponge haplotypes. Consensus with Haplotype A sequence is indicated by dots. Base insertions or deletions (indels) are indicated by dashes. A total of 55 variable sites were observed.

	Sequence Position																															
						1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	4
	1	2	2	4	6	7	8	8	0	0	1	2	3	3	3	3	3	4	7	7	8	9	9	9	1	1	4	7
Haplotype	4	5	9	5	8	9	0	1	6	8	6	9	0	1	3	5	9	1	6	7	2	6	7	9	0	9	3	6
A	C	A	C	A	G	T	C	G	A	C	T	T	T	T	C	C	C	-	C	A	T	C	A	T	-	A	G	T
B	T	G	T	G	A	C	T	T	G	T	C	-	-	-	.	T	T	C	T	C	G	-	-	.	A	C	A	C
C	.	.	.	.	.	.	.	.	.	.	C	-	-	-	T	.	.	-	.	.	.	.	.	C	-	C	.	.

	Sequence Position																															
	4	5	5	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	7	1	1	2	2	3	3	4	4	5	8	0	1	1	1	2	4	4	4	5	6	6	6	6	6	6	8
Haplotype	7	6	8	7	9	0	1	0	8	4	1	6	3	4	6	3	0	4	9	7	1	2	3	4	6	7	4
A	G	G	C	-	T	T	T	G	T	C	A	-	T	T	C	C	T	A	C	G	-	-	-	G	-	-	C
B	A	A	T	T	C	C	C	C	G	T	G	-	C	C	T	A	-	C	T	-	C	G	C	T	G	A	T
C	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	G	C	.	G	A	.

Table 6.2: Characterization of sequence variation among three ITS haplotypes observed in intertidal sponge populations. Pairwise comparisons of haplotypes with averages of all comparisons for base insertions/deletions (indels), transitions (t_i), transversions (t_v), and the ratio of transitions to transversions ($R = t_i / t_v$). An overall comparison of the three haplotypes is given. The diagram below the table indicates which base changes are transitions and transversions

Comparison	indels	t_i	t_v	R
A-B	15	28	9	3.1
A-C	9	3	1	3.0
B-C	8	29	8	3.6
Average	11	20	6	3.3
Overall	16	30	9	3.3



(node support = 100, Fig. 6.2). ITS sequences obtained from European samples of *Halichondria panicea* and *H. bowerbanki* grouped outside of the Alaska haplotype clade.

Haplotype frequency and distribution were correlated with habitat type (Table 6.3). Haplotype A was almost exclusively found among hard substrate populations (44 of 47 colonies) and haplotype C was the predominant type found in soft-sediment habitats (39 of 46 colonies). Haplotype B occurred only in soft-sediment populations and only at low frequencies.

Measures of diversity based on ITS haplotypes were calculated to characterize the four sponge populations (Table 6.4). Haplotype diversity ranged from zero at Point Pogibshi, where only a single haplotype was observed, to 0.391 at Jakolof Bay. Both Camel Rock and Seldovia Bay exhibited intermediate levels of haplotype diversity. Nucleotide diversity followed a similar pattern with the lowest value reported for Point Pogibshi and the highest at Jakolof Bay. A test of selective neutrality, Tajima's D, was not statistically significant for any of the populations. Nucleotide composition of the sequences averaged 28.01% G, 19.86% A, 25.37% T, and 26.77% C.

CO1 Sequences

Three major haplotypes were identified for the partial CO1 sequence and were designated CO1-A, CO1-B, and CO1-C. Haplotype A was found only in hard substrate samples and haplotypes B and C were exclusively soft-sediment

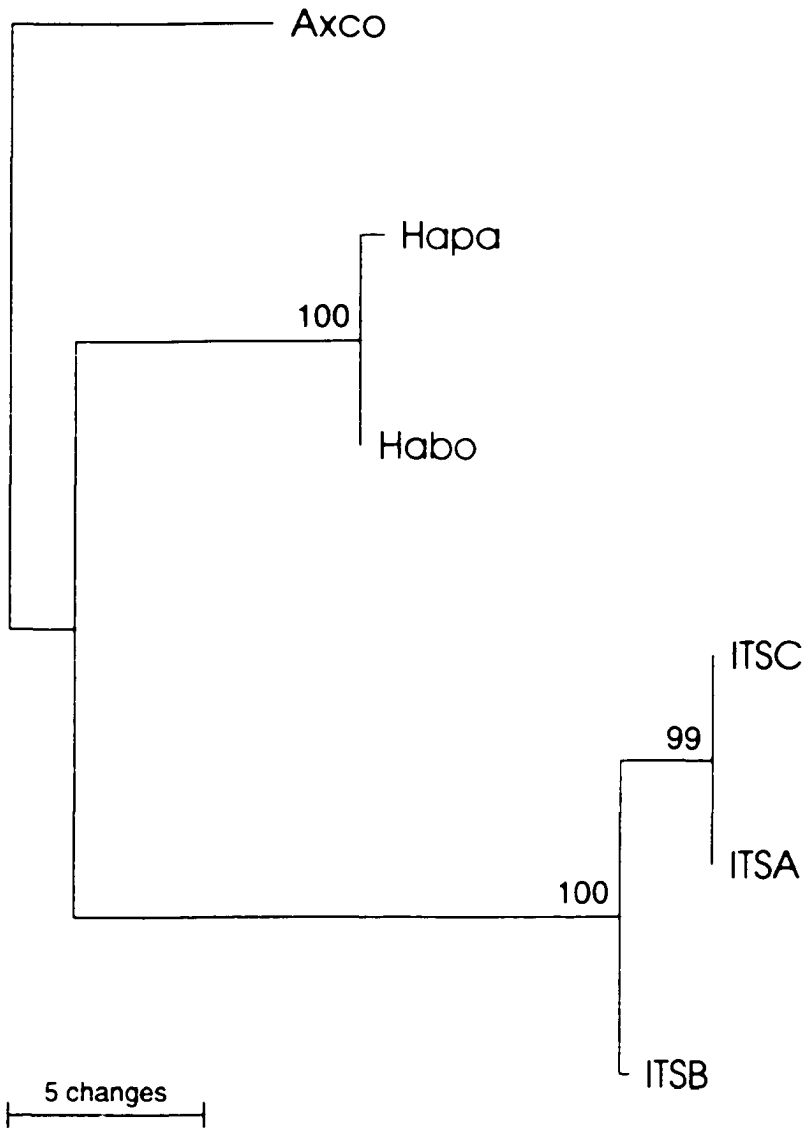


Figure 6.2: Single most parsimonious phylogenetic tree inferred by non-weighted parsimony methods based on ITS sequence data. Values at nodes indicate bootstrap values (50% majority, 1000 replications). Scale represents the number of base changes needed for the predicted tree. *Axinella corrugata* (Axco) was used as an outgroup. Hapa = *Halichondria panicea*, Habo = *Halichondria bowerbanki*.

Table 6.3: Frequency and distribution of three ITS haplotypes found among 93 sponge colonies sampled from Kachemak Bay populations. Hard substrate sites are Camel Rock (CR) and Point Pogibshi (PP), soft-sediment sites are Jakolof Bay (JB) and Seldovia Bay (SB).

	n per locality				
Haplotype	CR	JB	PP	SB	Total
A	19	0	25	1	45
B	0	5	0	1	6
C	3	19	0	20	42

Table 6.4: Diversity measures of three ITS haplotypes in four intertidal sponge populations. K = number of haplotypes per population, *h* = haplotype diversity, π = nucleotide diversity based on the Tamura-Nei distance method. Standard deviations are in parentheses. Tajima's D is a test of selective neutrality. ns = not statistically significant (α = 0.05).

Population	**K**	***h***	π	**Tajima's D**
Hard Substrate				
Camel Rock	2	0.247 (0.108)	0.048 (0.024)	2.148 ns
Point Pogibshi	1	0.000 (0.000)	0.000 (0.000)	0.000
Soft-Sediment				
Jakolof Bay	2	0.391 (0.091)	0.105 (0.052)	6.992 ns
Seldovia Bay	3	0.178 (0.106)	0.040 (0.020)	0.032 ns

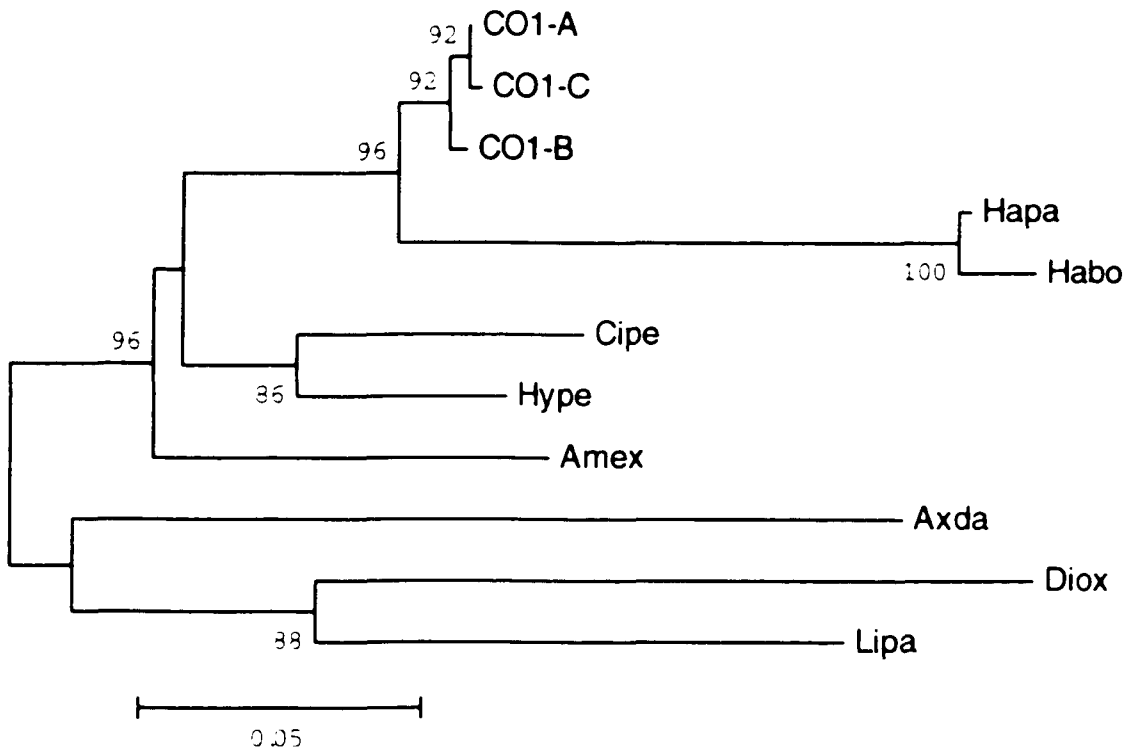
samples. Individuals categorized as CO1-A were also grouped in the ITS A haplotype. CO1-B individuals were also ITS B; CO1-C were ITS C. A cluster analysis of the CO1 haplotypes, including sequence data from several additional sponges species (Erpenbeck et al., 2002), was performed (Fig. 6.3). Trees generated from CO1 data were topologically similar to trees generated using ITS data.

Population Relationships

Pairwise comparisons of molecular differentiation based on ITS sequence data showed a statistically significant difference based on Φ_{ST}, an analog of F_{ST} for designed for haplotype data (Excoffier et al., 1992), among all population comparisons (Table 6.5), indicating a significant degree of sequence divergence. The number of predicted migrants between populations is low. Relationships among the sites, as determined by coancestry coefficient distances, show that same habitat sites are more closely related to one another than they are to sites in other habitats (Fig. 6.4). Camel Rock and Point Pogibshi grouped together, as did Jakolof Bay and Seldovia Bay.

Discussion

ITS haplotype distributions corresponded with habitat type. Haplotype A was found primarily among hard substrate samples and haplotypes B and C



Label	Order	Family	Genus	Species
Hapa	Halichondrida	Halichondriidae	*Halichondria*	*panicea*
Habo	Halichondrida	Halichondriidae	*Halichondria*	*bowerbanki*
Cipe	Halichondrida	Halichondriidae	*Ciocalypta*	*penicillus*
Amex	Halichondrida	Halichondriidae	*Amorphinopsis*	*excavans*
Diox	Halichondrida	Halichondriidae	*Didiscus*	*oxeata*
Axda	Halichondrida	Axinellidae	*Axinella*	*damicornis*
Lipa	Halichondrida	Dictyonellidae	*Liosina*	*paradoxa*
Hype	Halichondrida	Hymeniacidonidae	*Hymeniacidon*	*perlevis*

Figure 6.3: Cluster analysis of CO1 haplotypes. CO1-A corresponds to ITS haplotype A, CO1-B to ITS B, and CO1-C to ITS C. Numbers at nodes represent bootstrap values from 1000 iterations. Values less than 50 are not reported. Sequences data of listed species are from Erpenbeck et al., (2002).

Table 6.5: Pairwise comparisons of molecular differentiation based on ITS sequence data of four sponge populations. Differentiation between populations (Φ_{ST}) values are listed above the diagonal; number of migrants (N_m) below the diagonal. Analyses are based on the Tamura-Nei distance method. Asterisk (*) = statistically significant ($\alpha = 0.05$).

	Camel Rock	**Point Pogibshi**	**Jakolof Bay**	**Seldovia Bay**
Camel Rock		0.106*	0.295*	0.720*
Point Pogibshi	4.210		0.449*	0.894*
Jakolof Bay	1.197	0.613		0.613*
Seldovia Bay	0.194	0.059	0.315	

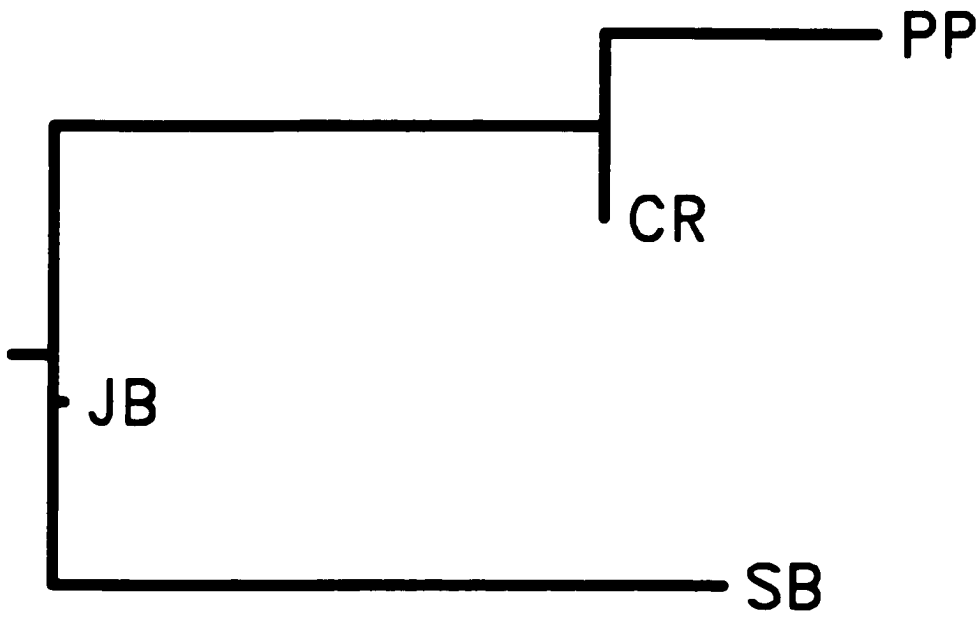


Figure 6.4: Neighbor-joining phylogeographic tree describing the relationships among sponge populations sampled at four sites in Kachemak Bay, Alaska. Branch lengths are relative and represent coancestry coefficient distances (Reynolds et al., 1983). Hard substrate habitats are Camel Rock (CR) and Point Pogibshi (PP). Soft-sediment locations are Jakolof Bay (JB) and Seldovia Bay (SB).

among soft-sediment samples. A phylogeographic tree of coancestry coefficients (Fig. 6.4) grouped the same habitat sites together and supported the hypothesis that haplotypes were correlated with habitat. Results from the CO1 sequence data were similar to ITS results and further supported the habitat-haplotype relationship. Two independent data sets, nuclear (ITS) and mitochondrial (CO1) DNA loci, suggest substantial genetic differentiation between sponge populations in hard substrate and soft-sediment habitats, as initially hypothesized from the analyses of microsatellite loci (see Chapter 5). While there is strong evidence the two taxa comprise species, it cannot be determined from this study whether or not separate species are associated with each habitat. Further investigation based on morphological, ecological, and genetic characteristics are needed to ascertain species composition.

For both ITS and CO1 sequence analyses, the Alaska haplotypes clustered as a sister group to European samples of *Halichondria panicea* and *H. bowerbanki* (Figs. 6.2, 6.3). Since *H. panicea* and *H. bowerbanki* were deemed the most likely species to be found in Kachemak Bay, it was expected that the Alaska samples would cluster with at least one of the two species. The results suggest that there is either substantial genetic differentiation within cosmopolitan species or, more likely, that the species in Alaska are not *H. panicea* or *H. bowerbanki*. Further, the long branch length for the *H. panicea*/*H.bowerbanki* cluster (Fig. 6.3) suggests that taxa more closely related to the Alaska samples

are missing from the analyses, and leads to the possibility that a completely different genus of sponges is found in Alaska.

Other studies on marine invertebrates (Table 6.6) examining variability within the ITS regions have found intraspecific differences ranging from 0% to 68% sequence difference and interspecific differences of 0% to 46%. Variation in the ITS regions appears to be taxa-specific and needs to be evaluated relative to similar taxa values. The three Alaska ITS haplotypes averaged 5.3% sequence divergence including insertions/deletions, transitions, and transversions (see Table 6.2), over the 700 bp fragment. Haplotypes A and C showed only 1.8% sequence difference between them, which is similar to the variability seen within *Axinella corrugata* (Porifera; Lopez et al., 2002). Larger differences were observed when either Haplotype A or C was compared to Haplotype B, with 7.4% and 6.4% sequence difference, respectively. These differences are in the range seen between *Axinella* spp. (Lopez et al., 2002). While the results of this study strongly suggest the need for a reassessment of sponge species found in southcentral Alaska, it must be noted that phylogeographic trees and cluster analyses are hypotheses of relationships and additional evidence is needed to validate the results. In addition, the relationships presented here are based on two gene trees and may not represent the 'true' evolution of the taxa involved. Nevertheless, a re-evaluation of species is needed to complement and validate ongoing ecological investigations of sponge populations along the Pacific shores of North America. Thus, many of

Table 6.6: Sequence divergence within and between species for several marine invertebrate species.

Organism	Intraspecfic Sequence Divergence	Interspecific Sequence Divergence	Study
Porifera			
Axinella corrugata	<1%	-	Lopez et al., 2002
Axinella spp.	-	6.4%	Lopez et al., 2002
Axinella/Ptilocaulis	-	10-12%	Lopez et al., 2002
Cnidaria			
Paracyathus stearnsii	0.7%	-	Beauchamp &
Balanophyllia elegans	4.7%	-	Powers 1996
Balanophyllia/ Paracyathus/Favia	-	27-38%	Beauchamp & Powers 1996
Acropora spp.	-	0-5.2%	Van Oppen et al., 2000
Rhodactis howesii	2.5%	-	Chen et al., 1996
Annelida			
Sabella spallanzanii	4%	-	Patti & Gambi 2001
Perinereis spp.	-	~30%	Chen et al., 2002
Mollusca			
Mya arenaria	0.7%	-	Caporale et al., 1997
Tridacna crocea	29-68%	-	Yu et al., 2000
Crustacea			
Eriocheir formosa	0.9-2.3%	-	Chu et at ., 2001
Eriocheir spp.	-	5.4-16.3%	Chu et at ., 2001
Penaeus japonica	18.1%	-	Chu et at ., 2001
Penaeus spp.	-	46.3%	Chu et at ., 2001

the questions raised in this study are outside its intended scope and remain for future investigation.

Summary

The results of this study have provided insights into the composition of sponge species inhabiting the waters of Kachemak Bay, Alaska. Based on historical identifications, a single species, *Halichondria panicea*, was the primary sponge species in the intertidal zone. Genetic evidence from this study suggests that at least two species are present, and their species designation may be in question. Distributions of ITS haplotypes corresponded to habitat type with haplotype A primarily occurring in exposed, hard substrate habitats and haplotypes B and C predominating at protected, soft-sediment locations. CO1 haplotype distributions supported this finding. Cluster analyses of the sequence data grouped the Alaska haplotypes as a sister group to European samples of *Halichondria panicea* and *H. bowerbanki*, suggesting that the species in Alaska may not be either of the two species. Further investigation of the taxonomic placement of sponge species in Alaska is needed.

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Chapter 7: Summary

Mechanisms structuring a rocky intertidal community in Kachemak Bay, Alaska, dominated by the encrusting sponge *Halichondria panicea*, were investigated through field and laboratory experiments.

The relationship between *Halichondria panicea* and overlying macroalgae was studied using experimental manipulations of permanent quadrats established on the study site. No effect of removing the macroalgae was observed on *Halichondria* abundance. Another experiment investigated the predator-prey dynmaics between *H. panicea* and its primary predator *Archidoris montereyensis* with a laboratory feeding trial. Results showed that nudibranchs consuming symbiotic sponge had higher feeding and egg production rates than individuals eating aposymbiotic sponge. Small nudibranchs (<60 g) had increasing growth rates but large nudibranchs (>60 g) exhibited decreasing growth rates. Finally, *H. panicea*'s response to a simulated predation event was examined. Initial sponge growth rates into experimental feeding grooves were high indicating an organismal response mechanism to tissue damage. Four weeks later, growth rates decreased to approximately normal, undisturbed growth rates.

Predation is a key structuring mechanism for some marine communities. Prey abundance may fluctuate with strength of predator recruitment and persistence except in cases where some of the prey population has a refuge in

space or time from predation. A natural experiment occurred when *Archidoris montereyensis*, a predatory nudibranch, recruited in high numbers into an established sponge population under investigation. Percent cover estimates show that *Halichondria panicea* averaged 53.4% ±9.9% cover from August 1994 through August 1996. Total numbers of *A. montereyensis* at the study site ranged from 12 to 42 from 1994-1996. In the spring of 1997, strong recruitment resulted in an average population of 156 *A. montereyensis* on site from May to July. Percent cover of *H. panicea* declined from 40% in May to 15% in July. By August 1997, sponge was absent at the study site and the number of nudibranchs declined to 7 individuals by September. As of June 2002, the site once dominated by *H. panicea* is open rock with heavy recruitment of annual macroalgae occurring. The predator-prey relationship of *A. montereyensis* and *H. panicea* is an example of a chase through space and time, with convergence resulting in extreme population fluctuations and an unstable community.

Based on genetic evidence (see below), two sponge species occurring in separate habitats likely comprise the intertidal populations investigated in Kachemak Bay. Until now, it was commonly accepted that a single species, *Halichondria panicea*, dominated intertidal habitats in the region. Subsequent ecological and biological studies described below have been revised to reflect this new view of the species composition of sponge populations.

The reproductive cycles of what now appears to be two species, *Halichondria panicea* and *H. bowerbanki*, were investigated. Two populations of

H. panicea from semi-exposed rocky habitats and two populations of *H. bowerbanki* from protected, soft-sediment bays exhibited seasonal peaks in oocyte production and maturation. Small oocytes were present throughout the year indicating a degree of reproductive investment at all times. Populations in exposed habitats produced embryos 3-4 months earlier than populations in protected habitats (November vs. March). Male:female sex ratios for all populations ranged from 1:3 to 1:8, coinciding with sex ratios reported for other *Halichondria* populations. A few simultaneous hermaphrodites, individuals containing both oocytes and spermatocysts, were observed and ranged from 2-8% of each sample population. Based on the frequency of occurrence of large oocytes and embryos, *H. panicea* populations at exposed habitats appear to rely on sexual reproduction as the primary mode of propagation, while *H. bowerbanki* populations in protected habitats may rely more on asexual modes of reproduction. These results for *Halichondria* spp. in southcentral Alaska contribute to our understanding of environmental influences on timing and mode of reproduction in sponges.

The genetic population structure of the four intertidal sponge populations, two from exposed, hard-substrate habitats and two from protected soft-sediment sites, was determined using microsatellite loci. Six variable and reliable microsatellite regions were isolated and characterized from a single sponge colony collected at a hard substrate site. Samples from the soft-sediment locations could only be amplified at three of the six loci and were dropped from

further analyses. Characterizations of genetic diversity were completed for the hard substrate populations. Based on F_{ST} values, the hard substrate sponge populations are significantly differentiated from one another. No significant inbreeding has occurred within either population and a bottleneck effect was not detected. None of the samples were genetically identical for the loci examined indicating that cloning is probably not occurring. Products of sexual reproduction appear to be maintaining the populations at their respective locations, though there is little gene flow between the hard substrate populations studied.

Further investigation of the genetic diversity of hard substrate and soft-sediment sponge populations was conducted. Results of DNA sequence analyses have provided insights into the composition of sponge species inhabiting Kachemak Bay. Based on historical identifications, a single species, *Halichondria panicea*, was the primary sponge species in the intertidal. Genetic evidence from this study suggests that at least two species are present, and their species designation may be in question. Distributions of ITS and CO1 haplotypes corresponded to habitat type with haplotype A primarily occurring in exposed, hard substrate habitats and haplotypes B and C predominating at protected, soft-sediment locations. Cluster analyses of the sequence data grouped the Alaska haplotypes as a sister group to European samples of *Halichondria panicea* and *H. bowerbanki* suggesting that the species in Alaska may not be either of the two known species.

Appendix A: Isolation and Characterization of Microsatellite Loci in the Intertidal Sponge *Halichondria panicea* (Porifera)[1]

Introduction

Marine sponges have historically received limited attention by ecologists, thus lagging behind in information relative to more commercially important or easily studied organisms. While research on the ecological and especially chemically-mediated roles of sponges has increased primarily due to studies of global climate change and pharmaceutical interests, studies on sponge population genetics still suffer from lack of appropriate genetic markers. Markers for mitochondrial DNA (mtDNA) are insufficient for population level work because they are generally not variable enough within a species to detect population differences. In addition, mtDNA markers cannot reflect an organism's complete genetic history, providing information about maternal gene flow only. Confounding the lack of genetic markers, morphological characteristics of sponges are often inadequate for species identification, as many traits are similar among species. Allozyme assays have been standard analytical techniques for

[1]Knowlton, A.L., B.J. Pierson, S.L. Talbot, and R.C. Highsmith. In preparation. Isolation and characterization of microsatellite loci in the intertidal sponge *Halichondria panicea* (Porifera). *Molecular Ecology Notes.*

species identification, but the requirement of freshly collected tissues is often logistically prohibitive.

In southcentral Alaska, four populations of the intertidal sponge *Halichondria panicea* were investigated with respect to their role in intertidal community structure. One of the goals of the research was to assess the level of genetic relatedness within and between the study populations and to infer the relative importance of sexual vs. asexual reproduction.

Microsatellite markers are considered to be the tool of choice for investigating relationships between populations, due to their high levels of polymorphism and presumptive selective neutrality (McDonald and Potts 1996, Goldstein and Schlötterer 1999). Here, we report primer sequences and amplification conditions for seven novel microsatellite loci in *Halichondria panicea*, and provide preliminary data on allelic variation of these loci in one of the intertidal sponge populations to assess their utility as high-resolution genetic markers to examine population relationships for this species.

Materials and Methods

Tissue samples were collected from intertidal sponge populations in Kachemak Bay, Alaska. Whole genomic DNA was isolated from ethanol preserved tissues following proteinase K digestion and a CTAB/PVP extraction protocol modified after Stewart and Via (1993). Genomic DNA extractions were

quantified using a DyNA Quant 200 Fluorometer (Amersham Biosciences, Piscataway, NJ) and diluted to 50 ng/μl working solutions.

Methods described in Kandpal et al., (1994) were used to isolate and characterize microsatellite repeat motifs using genomic DNA from a single sponge colony collected within a hard-substrate population at Camel Rock, Seldovia, Alaska. Genomic DNA was digested using *Sau*3A I enzyme (New England Biolabs, Inc., Beverly, MA). DNA fragments were separated via electrophoresis, stained with ethidium bromide and visualized under UV transillumination. Fragments ranging from 400-1500 bp and suitable for insertion into a vector were cut from the gel and extracted from the agarose using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). Purified products were quantified using fluorometry and ligated to *Sau*3A I linkers using T_4DNA ligase (New England Biolabs). After removal of excess *Sau*3A I linkers by electrophoretic fractionation in low-melt agarose gel, linker-ligated fragments were amplified using the polymerase chain reaction (PCR) to create a whole-genome PCR library. The *Sau*3A I linker-ligated PCR amplified products were then denatured and hybridized to two different biotinylated oligonucleotide probes [5-$(CA)_{15}$TATAAGATA-Biotin and 5-$(GA)_{15}$TATAAGATA-Biotin] (LIFECODES Corporation, Stamford, CT). Vectrex Avidin D matrix (Vector Laboratories, Burlingame, CA) was used to capture the hybridized fragments, and non-specifically bound DNA fragments were eliminated using stringent washes. Captured microsatellite-enriched fragments were eluted, washed and amplified

using PCR, and enrichment was visually confirmed using dot blots probed with alkaline phosphatase labeled oligonucleotides (Lumi-Phos-480 Hybridization Kit, LIFECODES, Stamford, CT). PCR fragments were ligated into a plasmid vector (pCR2.1® vector; Invitrogen, San Diego, CA) and cloned in *Escherichia coli* cells provided in the kit. Colony lifts were transferred and fixed to a charged Nylon membrane (Hybond N+, Amersham-Pharmacia), using alkaline transfer. Membranes were hybridized with $(CA)_n$ and $(GA)_n$ alkaline phosphatase labeled probes and positive colonies were identified through chemiluminescence and autoluminography (LIFECODES, Stamford, CT). Selected colonies were cultured in liquid media, purified, and sequenced using vector-specific primers and analyzed on a LI-COR Longread 4200 automated sequencer (Lincoln, NE). Primers flanking microsatellite repeats were designed for eight loci.

A total of seven oligonucleotide primer pairs flanking microsatellite repeats were next screened for variability (Table A.1). The forward primer for each primer pair was synthesized with an additional modified 19-bp tail (M13F-29 or M13R) added to the 5' end of the oligonucleotide (Steffens et al. 1993, Oetting et al. 1995) to facilitate fluorescent labeling and visualization of amplified fragments with a sequence complementary to the specific tail and directly labeled to the infrared fluorophore IRDye700 and IRDye800 (LI-COR, Lincoln, NE). PCR amplifications were carried out in a final volume of 10 µl and contained 50 ng genomic DNA, 0.2mM dNTPs, 1X PCR buffer (10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM

Table A.1: Description of seven microsatellite loci isolated from *Halichondria panicea*. Information given includes locus name, core repeat in sequenced clone, primer sequences, and name of tail sequence.

Locus	Repeat Motif	Primer Sequences (5'–3')	Tail[3]
Hapa21	$[(GT)_{4\text{-}6}GGGTGGA (C)_{3\text{-}6}YTCT]_{12}$	F[1]: ACCACACCTTCCCAGAGC R[2]: CAACACTGTAGAGTTTGC	M13F -29[4]
Hapa22	$(CA)_7$	F: TTATACTACTGTGCAAGC R: ATCACCATTCAGCCGGCTGC	M13F -29
Hapa24	$(CA)_{14}$	F: CTTGAGCAGCTGTAGCAG R: CTTATTATGCCTCGGAGC	M13F -29
Hapa28	$[CC(CA)_2C(CA)_4TA]_2CA$	F: TTTGGAGGCGTACCTTGAGC R: GCATATAAGTTAGCAGCG	M13F -29
Hapa37	$[(CA)_2TGA]_2(CA)_5CC (CA)_4N(CA)_4$	F: GCCGTTACAATCATACG R: TATACGAGTCACATGTGC	M13R[5]
Hapa45	$(CA)_{16}$	F: TAAGGGTCTTCAGTAATGC R: ATAGTACGATTACCCAAG	M13R
Hapa46	$(CA)_{14}$	F: CGGCACATACTGTGAAGC R: ATCAACAAGCCATCATGC	M13R

[1]F, forward primer
[2]R, reverse primer.
[3]Forward primer was tailed on the 5'– end for all loci.
[4]M13F -29 sequence (5'–3'): CACGACGTTGTAAAACGAC
[5]M13R sequence (5'–3'): GGATAACAATTTCACACAGG

KCl, 0.01% (w/v) gelatin), 0.1 μg/μl bovine serum albumin (Hapa24), 0.5 units Amplitaq DNA polymerase (PE Biosystems), 0.1 μM (Hapa21, Hapa28, Hapa37, Hapa46) or 0.15 μM (Hapa22, Hapa24) IRDye-labeled M13 tail primer, and 0.5 μM (Hapa24) or 1.0 μM (Hapa 21, Hapa22, Hapa28, Hapa37, Hapa46) of each forward and reverse primer. PCR reactions began with an initial denaturation at 94°C for 2 minutes followed by 40 cycles of 94°C for 15 s; locus-specific annealing temperature (Table A.2) for 15 s; 72°C for 30 s followed by a 30-minute extension at 72°C.

PCR products were electrophoresed and visualized on a 48-well, 25 cm, 6% polyacrylamide gel, on a LI-COR 4200L automated sequencer. Three individuals heterozygous at each locus were sized against a fluorescently-labeled M13 sequence ladder of known length and were included in subsequent genotyping gels as size standards.

Results

Of the seven loci screened, all were polymorphic in *Halichondria panicea*. The number of alleles per locus varied from 2 to 8, with a mean of 5.43 ± 0.92 (Table A.2). One locus (Hapa45) did not produce clear bands after several optimization attempts and was dropped from further characterization and analysis. Heterozygosity for the remaining six loci ranged from 0.14 to 0.68.

Table A.2: Characterization of microsatellite loci isolated from *Halichondria panicea*. Locus specific annealing temperature (T_a), number of alleles and size range in base pair (bp) at each locus, observed (H_O) and expected (H_E; Nei 1987) heterozygosities. Information based on clone population. na = data not available.

Locus	T_a[1] (°C)	No. of Alleles	Size Range (bp)	H_O	H_E
Hapa21	61-54	7	167-243	0.68	0.78
Hapa22	50	6	186-250	0.41	0.52
Hapa24	50	8	155-179	0.57	0.76
Hapa28	56	2	175-179	0.14	0.13
Hapa37	61-54	2	257-287	0.32	0.27
Hapa45	56	~7	~219-274	na	na
Hapa46	61-54	6	200-224	0.68	0.73

[1]Temperature range indicates touchdown cycling

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Appendix B: Microsatellite Database

Table B.1: Allele sizes at six microsatellite loci for sponge samples from four intertidal sponge populations in Kachemak Bay, Alaska. Two populations, Camel Rock (CR) and Point Pogibshi (PP), were from hard substrate habitats and two populations, Jakolof Bay (JB) and Seldovia Bay (SB), were from soft-sediment habitats. Data includes locus name (Hapa 21, Hapa 22, Hapa 24, Hapa 28, Hapa 37, Hapa 46), population designation, sample number, and allele sizes for each locus. Empty cells indicate no data available.

		Hapa 21		Hapa 22		Hapa 24		Hapa 28		Hapa 37		Hapa 46	
CR	2001-001	185	231	232	241	171	173	179	179	257	287	200	220
	2001-002	185	185	186	236	173	175	179	179	257	257	224	224
	2001-003	185	231	250	250	175	175	179	179	257	287	224	224
	2001-004	185	213	196	236	173	173	179	179	257	287	220	224
	2001-005	207	207	236	250	173	179	179	179	257	287	200	224
	2001-006	207	207	236	236	165	177	179	179	257	257	200	220
	2001-007	185	243	236	236	171	173	179	179	257	257	200	220
	2001-008	167	185	236	236	165	173	175	179	257	257	200	220
	2001-009	183	243	236	236	171	173	179	179	257	257	200	220
	2001-010	185	207	236	236	165	173	179	179	257	287	220	222
	2001-011	185	207	236	236	165	165	179	179	257	287	220	222
	2001-012	207	231	236	241	171	173	179	179	257	257	200	200
	2001-013	243	243	186	250	165	173	179	179	257	257	224	224
	2001-014	207	243	236	250	175	175	179	179	257	257	208	224
	2001-015	167	167	236	236			175	179	257	257	200	220
	2001-016	243	243	186	250	165	165	179	179	257	287	224	224
	2001-017	183	207	236	236	173	173	179	179	257	257	200	220
	2001-018	185	207	236	236	161	161	179	179	257	257	200	200
	2001-019	185	231	236	236	165	165	179	179	257	257	218	220
	2001-020	185	243	232	236	173	173	179	179	257	257	200	220
	2001-021	207	207	236	236	165	173	179	179	257	257	200	200
	2001-022	167	167	236	236	155	173	175	179	257	257	200	224

Table B.1 (cont.): Allele sizes at six microsatellite loci for sponge samples from four intertidal sponge populations in Kachemak Bay, Alaska.

		Hapa 21		Hapa 22		Hapa 24		Hapa 28		Hapa 37		Hapa 46	
PP	2001-071	207	207	240	250	175	189	179	179	257	257	200	220
	2001-072	232	232	241	250	175	175	179	179	257	287	200	224
	2001-073	185	185	236	250	175	175	179	179	257	287	220	224
	2001-074	185	207	241	250	161	177	179	179	257	287	200	220
	2001-075	185	185	228	236	173	173	179	179	257	287	200	224
	2001-076	207	207	236	241	163	175	179	179	257	287	200	224
	2001-077	207	207	232	241	173	175	179	179	287	287	224	234
	2001-078	207	231	236	241	165	175	179	179	257	287	224	231
	2001-079	183	183	232	250	173	173	179	179	257	257	200	200
	2001-080	207	207	236	236	173	173	179	179	257	257	200	200
	2001-081	185	185	248	250	173	173	179	179	257	287	220	224
	2001-082	207	232	236	236	173	179	179	179	257	287	200	218
	2001-083	185	232	226	236	179	179	179	179	287	287	200	231
	2001-084	232	232	226	241	165	165	179	179	257	289	200	234
	2001-085	207	207	236	241	175	175	179	179	287	287	218	233
	2001-086	185	207	250	250	173	175	179	179	287	287	200	224
	2001-087	207	231	226	236	175	175	179	179	257	257	224	224
	2001-088	185	231	229	236	173	173					222	224
	2001-089	185	207	236	236	163	179	179	179	257	287	218	220
	2001-090			229	236	173	173	179	179	257	257	208	224
	2001-091	183	183	236	250	175	175	179	179	257	257	224	224
	2001-092	183	185	236	241	173	179	179	179	257	257	218	224
	2001-093	183	185	229	241	165	175	179	179	257	257	200	224
	2001-094	207	232	252	252	163	171	179	179	257	257	218	218
	2001-095	185	207	255	255	173	175	179	179	257	257	200	234

Table B.1 (cont.): Allele sizes at six microsatellite loci for sponge samples from four intertidal sponge populations in Kachemak Bay, Alaska.

		Hapa 21		Hapa 22		Hapa 24		Hapa 28		Hapa 37		Hapa 46	
JB	2001-023							181	181				
	2001-024	167	167					175	193	287	287		
	2001-025	167	167					181	181				
	2001-026							181	181				
	2001-027	167	167					181	181	257	287		
	2001-028	167	167					175	193				
	2001-029	167	167					181	193	287	327		
	2001-030	167	167					181	181	257	257		
	2001-031	167	167					181	181	257	257		
	2001-032	167	167					181	181	257	287		
	2001-033	167	167					181	181	257	257		
	2001-034	167	167					175	193	257	257		
	2001-035							181	181	257	257		
	2001-036							175	193	257	257		
	2001-037	167	167					175	193	257	257		
	2001-038	167	167					181	181				
	2001-039	167	167					181	181	257	257		
	2001-040	167	167					181	181	257	257		
	2001-041							181	181	255	255		
	2001-042	167	167					181	181				
	2001-043							181	181	257	257		
	2001-044	167	167					175	193	257	287		
	2001-045							175	175	257	257		
	2001-046							181	181	257	257		

Table B.1 (cont.): Allele sizes at six microsatellite loci for sponge samples from four intertidal sponge populations in Kachemak Bay, Alaska.

		Hapa 21		Hapa 22		Hapa 24		Hapa 28		Hapa 37		Hapa 46	
SB	2001-047							175	175	257	257		
	2001-048							181	181	257	277		
	2001-049	167	167					175	175	257	257		
	2001-050	207	231					175	175	257	257		
	2001-051	167	167					175	175	257	257		
	2001-052	167	167					175	175	257	287		
	2001-053	207	207					179	179	257	287		
	2001-054	161	231					175	175	257	257		
	2001-055	167	167					175	175	257	277		
	2001-056							181	181	257	257		
	2001-057							181	181	257	287		
	2001-058							175	175	257	277		
	2001-059	161	161					175	175	257	287		
	2001-060	231	231					181	181				
	2001-061									257	257		
	2001-062	161	161					175	175	257	257		
	2001-063	161	161					175	175				
	2001-064	231	231					181	181				
	2001-065							181	181	257	267		
	2001-066	161	161					175	175	257	277		
	2001-067	207	207					175	175	257	257		
	2001-068							181	181	257	257		
	2001-069	161	161					175	175	257	267		
	2001-070							175	175	257	267		

Appendix C: ITS and CO1 Sequence Data

Table C.1: Sequence data for three sponge ITS haplotypes from Kachemak Bay, Alaska. ITS and rRNA-coding regions are indicated. Consensus with Haplotype A is indicated by a dot. Insertion or deletion of a base is indicated by a dash. Fragment length is 718 base pairs.

```
      18S rRNA ──────────────────────────────────────────────────────────►
ITSA  GGTTTAGTGA GAACTTTGGA CTGGAACTCT CTTGTTCAGC AATGAACGAG AGAGAGCCCG
ITSB  .......... ...T...... ....G...T. .......... ....G..... ..........
ITSC  .......... .......... .......... .......... .......... ..........

      18S rRNA ──────────────────────────────────────────────────────────►
ITSA  GGAAGCCGTT CTAACTGTAT CATTTAGAGG AAGTAAAAGT CGTAACAAGG TTTCCGTAGG
ITSB  ........A. .......... .......... .......... .......... ..........
ITSC  .......... .......... .......... .......... .......... ..........

      18S rRNA ──────────►| ITS-1 ──────────────────────────────────────►
ITSA  TGAACCTGCG GAAGGATCAT TACTGATTGG CTTTTCGGCC CTTCTCTGTG CACCGTTTTC
ITSB  .......... .......... ..|....... .......... .......... .........CT
ITSC  .......... .......... ..|....... .......... .......... ..........

      ITS-1 ──────────────────────────────────────────────────────────►
ITSA  GGTTCGGTCG AGCGTGTCTC GGCCGAGCGG GGGTTTGCCA GGCTCTCTTT TCCCCCCCCG
ITSB  T......... .......... .....G.T.. .....C.... ..........- -...T...T.
ITSC  .......... .......... .......... .....C.... ..........- -.T.......

      ITS-1 ──────────────────────────────────────────────────────────►
ITSA  -GGTGGGAGAG TGGGTGGCGA TCGAAGCCGG GCTCCCAGTC CTCCCGCGCC CCGTACATTT
ITSB  C......... .......... .......... .....TC... .G........ .....--...
ITSC  -......... .......... .......... .......... .......... .........C.

      ITS-1 ──────────────────────────────────────►| 5.8S rRNA ──►
ITSA  TTTTCAAAA- CACTTTGTAT TTTTTTCTCG TGAAAACTTA AAGTTTAAAC AACTTCTAAC
ITSB  .........A .........C. .......... .......... ..A....... ..........
ITSC  .........- .........C. .......... .......... .......... ..........

      5.8S rRNA ──────────────────────────────────────────────────────────►
ITSA  GGTGGACCCC TCGGCTCGTG CATCGATGAA GAACGCAGCA AACTGCGATA TGTAGTGTGA
ITSB  .......... .......... .......... .......... .......... ..........
ITSC  .......... .......... .......... .......... .......... ..........
```

Table C.1 (cont.): Sequence data for three sponge ITS haplotypes from Kachemak Bay, Alaska.

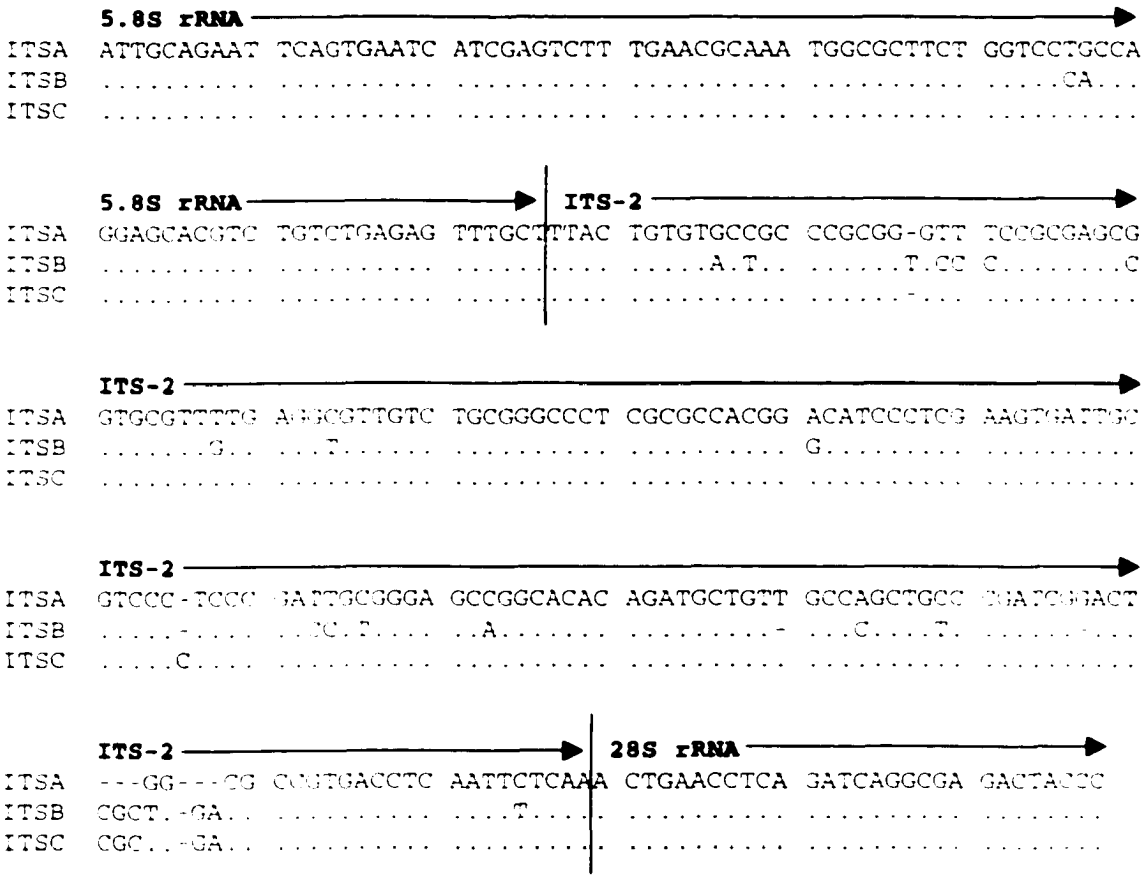

Table C.2: Sequence data of a partial CO1 fragment for three sponge haplotypes from Kachemak Bay, Alaska. Consensus with Haplotype A is indicated by a dot. Fragment length is 474 base pairs.

```
CO1-A GCTAAAAAAC AAATATTTGG TTATCTTGGT ATGGTTTATG CGATAGTATC AATAGGTGTA
CO1-B .......... .......... .........A .......... .......... ..........
CO1-C .......... .......... .......... .......... .T........ ..........

CO1-A TTAGGGTTTA TCGTATGAGC TCATCACATG TTTACAGTAG GGATGGATGT AGACACTAGA
CO1-B .......... .......... .......... .......... .......... ..........
CO1-C .......... .......... .......... .......... .......... ..........

CO1-A GCATATTTCA CTGCGGCAAC AATGATTATT GCAGTTCCTA CGGGAATAAA AATCTTTAGT
CO1-B .......... .......... .......... .......... .......... ..........
CO1-C .......... .......... .......... .......... .......... ..........

CO1-A TGAGTAGCCA CTATGTTTGG GGGTTCACTA CGATTAGACA CTCCTATGCT TTGGGCCATT
CO1-B .......... .......... .......... .......... .......... ..........
CO1-C .......... .......... .......... .......... .......... ..........

CO1-A GGGTTTGTAT TTTTATTTAC AGTAGGTGGT TTAACTGGGG TTGTTTTAGC GAATAGCTCT
CO1-B .......... .......... .......... .....G.... .......... ..........
CO1-C .......... .......... .......... .......... .......... ..........

CO1-A TTAGATATTG TTTTACATGA TACATATTAT GTGGTAGCAC ACTTCCATTA TGTTTTATCT
CO1-B .......... .......... .......... .......... .......... ..........
CO1-C .......... .......... .......... .......... .......... ..........

CO1-A ATGGGTGCCG TTTTCTCTTT GTTTGCTGGT TTCTATTATT GATTTGGAAA AATCACTGGT
CO1-B .......... .......... .......... .......... .......... ..........
CO1-C .......... .......... .......... .......... .......... ..........

CO1-A TATAGCTATA ACGAGGTTTA TGGAAAAATT CATTTTTGAA TAATGTTTAT TGGT
CO1-B .....T.... .......... .......... .......... .......... ....
CO1-C .......... .......... .......... .......... .......... ....
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